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Chronic stress exacerbates neuropathic pain via the integration of stress-affect–related information with nociceptive information in the central nucleus of the amygdala

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Abstract

Exacerbation of pain by chronic stress and comorbidity of pain with stress-related psychiatric disorders, including anxiety and depression, represent significant clinical challenges. However, the underlying mechanisms still remain unclear. Here, we investigated whether chronic forced swim stress (CFSS)–induced exacerbation of neuropathic pain is mediated by the integration of stress-affect–related information with nociceptive information in the central nucleus of the amygdala (CeA). We first demonstrated that CFSS indeed produces both depressive-like behaviors and exacerbation of spared nerve injury (SNI)-induced mechanical allodynia in rats. Moreover, we revealed that CFSS induces both sensitization of basolateral amygdala (BLA) neurons and augmentation of long-term potentiation (LTP) at the BLA-CeA synapse and meanwhile, exaggerates both SNI-induced sensitization of CeA neurons and LTP at the parabrachial (PB)-CeA synapse. In addition, we discovered that CFSS elevates SNI-induced functional up-regulation of GluN2B-containing NMDA (GluN2B-NMDA) receptors in the CeA, which is proved to be necessary for CFSS-induced augmentation of LTP at the PB-CeA synapse and exacerbation of pain hypersensitivity in SNI rats. Suppression of CFSS-elicited depressive-like behaviors by antidepressants imipramine or ifenprodil inhibits the CFSS-induced exacerbation of neuropathic pain. Collectively, our findings suggest that CFSS potentiates synaptic efficiency of the BLA-CeA pathway, leading to the activation of GluN2B-NMDA receptors and sensitization of CeA neurons, which subsequently facilitate pain-related synaptic plasticity of the PB-CeA pathway, thereby exacerbating SNI-induced neuropathic pain. We conclude that chronic stress exacerbates neuropathic pain via the integration of stress-affect–related information with nociceptive information in the CeA.

Keywords: Chronic stress, Pain exacerbation, Amygdala, GluN2B-containing NMDA receptor, Stress-induced hyperalgesia

1. Introduction

Pain has a strong emotional component, and chronic pain is frequently associated with affective disorders, such as anxiety and depression.[31,60,75] Negative emotions, however, can exacerbate chronic pain.[3,109] It is recognized that chronic stressful events induce biochemical, physiological, and psychological changes, causing stress-related mood disorders, including anxiety and depression,[18,28,56,62] which can increase the risk of developing chronic pain or exacerbate it.[25,60,109] For example, repeated exposure to a homotypic stressor such as forced swimming (FS) enhances formalin-induced inflammatory or thermal hyperalgesia in rats.[46,86,87,101–103] Repeated FS stress also enhances complete Freund adjuvant–evoked thermal hyperalgesia and mechanical hyperalgesia.[41,42] Yet, whether and how chronic forced swim stress (CFSS) exacerbates neuropathic pain are largely unknown.

The amygdala with its well-documented role in stress/emotion processing and related disorders, such as anxiety, depression, and persistent pain,[53,74,111] strongly supports the concept that the amygdala is a key player in the stress/emotional modulation of chronic pain.[69,100,107] The amygdala consists of several nuclei, among those the lateral (LA)/basolateral (BLA), and the central nuclei (CeA) are particularly important for sensory and emotion processing.[70,78] The latero-capsular part of the CeA (CeLC), which is now defined as the "nociceptive amygdala" because of its high content of nociceptive neurons,[74] receives nociceptive-specific information directly from the parabrachial (PB) area through the spino-parabrachio-amygdaloid pain pathway.[5,30] The LA and BLA receive polymodal sensory information (including stress and nociceptive input) from thalamic and cortical areas.[62,70,78] Highly integrated stress-affective–related information is then transmitted to CeA, the output nucleus for major amygdala functions,[6,55] to modulate the pain-related behaviors.

Additionally, pain-related synaptic plasticity and sensitization of CeLC neurons have been shown in animal models of arthritic,[72,73] visceral,[38] and neuropathic pain.[40,52] Pain-related plasticity in the CeLC is believed to be the consequence of

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converging stress-affect–related input from the BLA with nociceptive input from the spinal cord and brainstem.[70,74] Contribution of postsynaptic GluN2B-containing NMDA (GluN2B-NMDA) receptors to pain-related synaptic plasticity in the CeA has been found in animal model of arthritis pain.[7,49,58] Accumulating evidence implicates a role for GluN2B-NMDA receptors in amygdaloid synaptic plasticity[24,64,67,99] and chronic pain development.[61,85,117]

We hypothesized that chronic stress potentiates synaptic efficiency of the BLA-CeA pathway, leading to the activation of GluN2B-NMDA receptors and sensitization of CeA neurons, which subsequently facilitate pain-related synaptic plasticity of the PB-CeA pathway, thereby exacerbating nerve injury–induced neuropathic pain. In this study, we investigated whether pre-exposure of CFSS, which has been shown may robustly induce depressive-like behaviors in rats,[76,82,83] would exacerbate spared nerve injury (SNI)-induced pain allodynia. Furthermore, we examined whether the integration of potentiated BLA-CeA synapses induced by CFSS with enhanced PB-CeA synapses induced by SNI would underlie the CFSS-mediated exacerbation of neuropathic pain in rats subjected to SNI surgery.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 150 to 180 g at the beginning of the experiment were provided by the Department of Experimental Animal Sciences, Peking University Health Science Center. The rats were housed in separated cages with free access to food and water. The room temperature was kept at 24 ± 1°C under natural light–dark cycle. All animal experimental procedures were carried out in accordance with the guidelines of the International Association for the Study of Pain[118] and were approved by the Animal Care and Use Committee of Peking University. The behavioral experimenters were kept blind from the groupings of the rats.

2.2. Chemicals and antibodies

Ro 25-6981 [(R-(R*, S*)-a-(4-hydroxyphenyl)-b-methyl-4-(phenylmethyl)-1-piperidine propanol] (Tocris Bioscience, Bristol, United Kingdom) was dissolved in sterile 0.9% saline solution as a 1 mM stock solution, stored at −20°C, and diluted to desired concentrations (10 μM) just before experiments. Ifenprodil (Sigma-Aldrich, Saint Louis, MO) was dissolved in sterile 0.9% saline solution and was intraperitoneally (i.p.) delivered to rats at 3 mg·kg^{-1} body weight. In other experiments, it was dissolved in sterile dimethyl sulfoxide as a 20 μg·μL^{-1} of stock solution and was diluted to the working concentration of 1 μg·μL^{-1} and 10 μg·μL^{-1} with Tween–saline solution (5% dimethyl sulfoxide with 5% Tween 80 and 90% saline) for intra-CeA and intra-BLA microinjection, respectively. Imipramine (Sigma-Aldrich) was dissolved in sterile 0.9% saline or in Tween–saline solution for respective intraperitoneal (10 mg·kg^{-1} body weight) and intra-BLA (30 μg·μL^{-1}) application. Indomethacin (Alfa Aesar, Ward Hill, MA) was dissolved in sterile Tween–saline solution and delivered to rats at both 5 and 10 mg·kg^{-1} body weight by intraperitoneal administration.

Polyclonal rabbit anti-rat GluN1, rabbit anti-rat GluN2A, and mouse anti-rat GluN2B antibodies were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and horseradish peroxidase–conjugated secondary antibodies, including goat anti-rabbit IgG and goat anti-mouse IgG, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Chronic forced swim stress procedure

The FS test, also known as the behavioral despair test, is a well-established stress rodent model used to predict the clinical efficacy of antidepressant drugs.[21,80,81,114] It is now proved that chronic FS can also be used as an effective tool for inducing cognitive impairments analogous to those observed in depression and in other neuropsychiatric disorders.[10] In this study, CFSS was chosen as the mixture of both psychological (novelty, water) and physical (swimming exercise, temperature), naturalistic, mild stressor for identifying different pathways of coping with an unavoidable situation.[9,22,46] Generally, for short-term stress, most repeated or subchronic FS paradigm to induce depressive-like behaviour involves a 2 or 3 consecutive days of FS protocol.[41,80,87,96,97,102] In the present study, to investigate effects and the underlying mechanisms of chronic stress on neuropathic pain, we used a long-term exposure of FS protocol (ie, once per day in 15-minute sessions for 14 consecutive days) for chronic stress, which also has been widely used to induce depressive-like behaviour in previous studies.[22,76,83,97] Briefly, the rats were placed in a glass cylinder (45 cm high, 20 cm in diameter) filled with clear, fresh water (22°C-24°C) up to a height of 30 cm. An FS procedure was conducted to rats individually once per day in 15-minute sessions, continued for 14 consecutive days at random time to avoid the animals' adapting. Control rats were subjected to a sham swimming (sham FS) session by allowing them to wade in the cylinder that contained only 2 ± 4 cm of warm water. Rats were allowed to dry in a warm environment (30 ± 33°C) after each swimming session. The water was changed and the container was thoroughly cleaned for each animal. After 14 consecutive days of CFSS or sham FS, the SNI surgery was performed following the assessment of anxiety-like and depressive-like behaviors.

2.4. Measurement of body weight

Rats were weighed on the day before CFSS and then were repeatedly weighed on days 5, 10, and 15 post-CFSS, respectively, to calculate the body weight gains during days 1 to 5, days 5 to 10, and days 10 to 15 post-CFSS. Weight change was calculated by subtracting the body weight of the animal from the initial weight on the day before CFSS, while a positive number would suggest weight increase, and a negative number would indicate body weight decrease.[43]

2.5. Spared nerve injury surgery

Animals were anaesthetized i.p. with chloral hydrate (0.3 g·kg^{-1}), and the SNI procedure comprised a ligation, and distal axotomy of the tibial and common peroneal nerves was performed on the left thigh of rat as described in previous report.[23] Briefly, the skin of the lateral surface of the thigh was incised and a longitudinal section was made directly through the biceps femoris muscle exposing the sciatic nerve and its 3 terminal branches (the sural, common peroneal, and tibial nerves). The common peroneal and the tibial nerves were tight-ligated with 5-0 silk suture and sectioned distal to the ligation, removing 2 to 4 mm of the distal nerve stump. The sural nerve was left intact. Muscle and skin were closed in 2 layers. Sham-operated (sham SNI) animals underwent identical surgical procedures except that the tibial and common peroneal nerves were left intact. Any rats exhibiting

motor deficiency or lack of tactile allodynia were excluded from the study.

2.6. Behavioral testing

2.6.1. Sucrose preference test

Sucrose preference test (SPT) was performed as previously described.[104] Briefly, rats were habituated to 2 bottles of 1% sucrose solution for 48 hours at the beginning of the experiment, during which 2 bottles of 1% sucrose solution were placed in each cage. After adaptation, rats were deprived of water for 12 hours, followed by the sucrose preference test, in which the rats were housed in individual cages for 4 hours and had free access to 2 bottles that contained 1% sucrose or tap water. The position of the 2 bottles was varied every 2 hours during the test. At the end of 4 hours, sucrose solution and water consumption (in mL) was measured, and sucrose preference (%) was calculated as the ratio of sucrose consumption to sucrose plus water consumption. The SPT was carried out on the day before CFSS (day 0) and then on the day after last exposure of rats to CFSS (day 15).

2.6.2. Forced swim test

Twenty-four hours after the last exposure of CFSS, rats were forced to swim for 6 minutes as described in aforementioned methods and behaviors were monitored by video camera for subsequent analysis. The rats were considered immobile when they ceased struggling and remained floating motionless in the water, with only movements necessary to maintain their heads above water. The relative amount of time spent immobile was recorded by an expert observer blind to the experimental conditions. The duration of immobility was recorded during the last 4 minutes of the 6-minute testing period.

2.6.3. Elevated plus-maze test

Anxiety-like behavior was evaluated by elevated plus-maze (EPM) test on day 2 after the last exposure of rats to CFSS as described in our previous study.[52] Rats were placed at the center of the EPM consisting of 2 open and 2 closed arms (48 × 8 × 40 cm each arm) (Shanghai Mobiledatum information Technology Co, Ltd, Shanghai, China) and allowed to explore randomly for 5 minutes. The number of arm entries and the time spent in each arm were recorded during a 5-minute test period. The percentage of the time spent in open arms and the number of open arm entries were inversely related with anxiety-like behavior. The number of total arm entries was used as an index of locomotor activity. The EPM was carefully cleaned with 10% ethanol before each animal was placed on the equipment.

2.6.4. Assessment of mechanical allodynia

Mechanical allodynia, as a behavioral sign of neuropathic pain, was assessed by measuring paw withdrawal threshold (PWT) as described in previous reports.[23,105] In brief, animals were placed on a metal mesh floor covered with an inverted clear plastic cage (18 × 8 × 8 cm) and the plantar surface of the paw was stimulated with a series of ascending force von Frey monofilaments (Stoelting, Wood Dale, IL). Eight von Frey monofilaments with approximately equal logarithmic incremental (0.224) bending forces were chosen (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g). The filaments were applied perpendicular to the skin and depressed slowly until they bent. The correct angle of application was easily achieved on the mid-plantar site. The von Frey filaments were tested in order of increasing stiffness, with each applied 5 times at intervals of 1 to 4 seconds to slight different loci within the area being tested. The first filament in the series that evoked at least 1 response (from the 5 applications) was designated the threshold. The criterion response was a reflexive withdrawal that was clearly not related to stepping. The threshold was taken as the lowest force that evoked a brisk withdrawal response to 1 of 5 repetitive stimuli.[23] The lateral and medial plantar surface as well as the dorsal surface of the paw was tested randomly.

2.6.5. Assessment of locomotor function

Inclined-plate test was used for the assessment of locomotor function. Animals were placed crosswise to the long axis of an inclined plate. The initial angle of the inclined plate was 50°. The angle was then adjusted in 5° increments. The maximum angle of the plate on which the rat maintained its body position for 5 seconds without falling was determined according to the method reported by Rivlin and Tator.[91]

2.7. Implantation of intracranial cannula and drugs injection

Under a general anesthesia with chloral hydrate (0.3 g·kg^{-1}, i.p.), animals were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) with flat-skull position. A midline incision was made and the skin and underlying periosteum were retracted. Then, a stainless steel guide cannula (22-gauge; Plastics One Inc, Wallingford, CT) was implanted 0.5 mm above the right amygdale. The coordinates for the BLA and CeA were as follows with flat skull position and bregma as the reference: BLA, anterior, −2.8 mm; lateral, ±5.0 mm; ventral −6.5 mm; from Bregma[65]; CeA, 2.2 mm posterior to the bregma, 4.2 mm lateral from midline, and 8.1 mm ventral relative to the dura.[77] The guide cannula was fixed to screws in the skull using a mixture of acrylic and dental cement, and a stainless steel stylet was inserted into the cannula to maintain patency before microinjection. All animals were allowed to recover for 1 week before behavioral testing began.

Intra-BLA/CeA injection was given by lowering a 27-gauge infusion cannula to extend 1 mm beyond the tip of the guide cannulae to the site of injection. The injection cannula was attached with a polyethylene tube to a 1-μL Hamilton syringe. Infusions of drugs or an equivalent volume of vehicle into the BLA/CeA were carried out over 60 seconds, and the injection cannula was left in place for an additional 60 seconds to facilitate diffusion of the drugs from the tip of the injection cannula. To verify the cannula placement, 0.5 μL of black India ink was injected into the CeA immediately before the rat was euthanized at the end of each experiment, and ink diffusion into the BLA/CeA was histologically evaluated. Animals with wrongly placed cannulae were excluded from analysis.

2.8. In vivo electrophysiology: extracellular single-unit recording

2.8.1. Surgical preparation

Rats were initially anesthetized with urethane (1.2-1.5 g·kg^{-1}, i.p.) and then a cannula was inserted into the trachea for artificial respiration and to measure end-tidal CO_2 levels. A catheter was placed in the right jugular vein for continuous administration of anesthetic and for fluid support with Tyrode solution containing (in mM) NaCl 137, KCl 2.7, $CaCl_2$ 1.4, $MgCl_2$ 1.0, $NaHCO_3$ 6.0,

NaH_2PO_4 2.1, and D-(+)-glucose 6.5, pH 7.4, at a rate of 1.5 to 2 mL·h^{-1}. The animals were then placed in a stereotaxic apparatus, and a craniotomy was performed to allow the penetration of recording and stimulating electrodes. Incisions were made along the scalp to expose the skull, and burr holes were drilled and the dura was removed overlying the right BLA, CeA, or external capsule (EC) according to experiment need. Coordinates for these areas were determined using a stereotaxic atlas[77] as follows: BLA, 1.5 to 3.4 mm posterior to the bregma, 4.4 to 6.0 mm lateral from midline, and 7.6 to 8.4 mm ventral relative to the dura; CeA, 2.0 to 2.2 mm caudal to the bregma, 3.8 to 4.2 mm lateral from midline, and depth of 7 to 9 mm; EC, 2.2 to 4.0 mm rostral to the bregma, 3.5 to 4.0 mm lateral from midline, and depth of 3.4 to 5.0 mm. Bipolar, concentric stimulating electrodes were lowered into the right EC and CeA when recording the electrically evoked responses of the BLA neurons, and the recordings began at least 30 minutes after lowering of the stimulating electrodes. After surgical preparation, the animal was artificially ventilated with a small animal ventilator and paralyzed with curare (2.0 mg·kg^{-1}, i.v.). Constant levels of anesthesia and paralysis of the musculature were maintained by intravenous infusion of a mixture of urethane (0.1-0.15 g·kg^{-1}·h^{-1}) and curare (0.2 mg·kg^{-1}·h^{-1}) during the whole experiment. The depth of anesthesia was assessed by regularly testing the corneal blink and hind paw withdrawal reflexes and by continuously monitoring the end-tidal CO_2 levels (3.5%-4.5%), heart rate (330-460 beats/min), and electrocardiogram pattern. The rectal temperature was maintained at 36.5°C to 37.5°C via a feedback-controlled under-body heating pad.

2.8.2. Extracellular recordings

Extracellular recordings were made from single neuron in the right BLA or CeA with 2 to 5 MΩ parylene-coated tungsten microelectrodes (Friedrick Haer & Co, Bowdoinham, ME), driven by a micro-stepping motor. The recorded signals were amplified with an AC pre-amplifier, filtered with a passing band width of 0.5 to 1 kHz, displayed on an oscilloscope, and fed to a Pentium computer via a CED 1401 interface for online and offline analyses using the Spike2 software (Cambridge Electronic Design, Cambridge, United Kingdom). Single-unit recording was ensured on the basis of amplitude and shape of the action potentials (with a signal-to-noise ratio greater than 3:1, and a minimal duration of 1.0 milliseconds was set to exclude spikes that were not of somatodendritic origin).[92,93] Once an individual neuron was identified and its spike size was optimized, a receptive field in the hind limb was carefully searched, and the size and threshold of its total receptive field in the deep tissue and skin were determined. Only those neurons were included in this study whose spike configuration remained constant and could be clearly discriminated from background activity throughout the experiment.

All CeA neurons selected for this study were multireceptive (MR) neurons, which had a receptive field in the hind limb and responded consistently to innocuous stimuli but were more strongly activated by noxious stimuli.[37,50] An MR neuron and its receptive fields in the left hind limb were searched using the following stimuli as described in previous studies[71,72]: *Brush* (brushing the skin with a soft writing brush in a mild manner, which was considered innocuous because it did not evoke hind limb withdrawal reflexes in awake rats and was not felt painful when applied to the experimenters), *Press* (pressing a fold of skin with smooth forceps, which is marginally painful when applied to the skin in humans), and *Pinch* (pinching a fold of skin with toothed forceps, which was considered noxious because it evoked hind limb withdrawal reflexes in awake rats and was also felt painful when applied to the experimenters). An excitatory response was defined as the spike frequency it causes was higher than the upper 95% confidence interval of background activity. In each experiment, background activity of 1 CeA neuron was recorded for a minimum of 10 minutes as baseline and expressed as spikes per second. The most responsive site of the receptive field was then stimulated using mechanical test stimuli of innocuous (brush) and noxious (pinch) intensities to measure stimulus–responses relationships. Each stimulus was applied repeatedly for a duration of 15 seconds followed by a 15-second interval.

Neurons within the BLA selected for this study were output neurons projecting to the CeA. A projection neuron was identified by antidromic spikes elicited by the stimulation of the CeA. An antidromic response was defined as the ability of evoked spikes to follow stimulation frequencies of >250 Hz, displayed constant response latency, display collision with spontaneously occurring spikes when possible, and be evoked from an area receiving BLA projections.[13,92] In most cases, a test for collision of evoked and spontaneous spikes was not feasible due to the paucity of spontaneous spiking in many antidromically activated units. After searching a projection neuron, background activity of the BLA neuron was recorded for a minimum of 10 minutes as baseline and stimulus–responses relationships were measured by applying mechanical test stimuli of brush, smooth forceps, and toothed forceps to the most responsive site of the receptive field in the left hind limb (15-second duration each; 15-second intervals).

After recording of spontaneous activity, BLA neurons responsive to EC stimulation were isolated using a search-stimulate protocol as previously described.[13] Single-pulse electrical stimuli (0.2-0.7 mA, 0.2-ms duration, 0.5 Hz) were delivered to the EC while the recording electrode was lowered through the BLA to identify responsive neurons. Single units were operationally defined as orthodromic monosynaptic if they had an onset latency of <20 milliseconds, showed very little shift in latency when increasing the stimulus intensity, yet they showed some range (1-2 ms) in latency variability, and did not follow high-frequency stimulation (HFS >250 Hz), ruling out antidromic activation. Then, a train of 10 test stimuli (0.2-ms duration, 0.5 Hz, with a current intensity of 2× rheobase) was applied repeatedly to the EC at 5-minute intervals for up to 12 trials, and poststimulus histograms from the responses of the neuron were generated by the Spike2 software (Cambridge Electronic Design).

2.9. In vitro electrophysiology

2.9.1. Amygdala slice preparation

After decapitated, the rat brain was quickly dissected out and blocked in cold (4°C) artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, and 25 glucose. The ACSF was oxygenated and equilibrated to pH 7.4 with a mixture of 95% O_2 and 5% CO_2. Coronal brain slices (300 μm thick) containing the amygdale were prepared using a vibrating microtome (Leica Instruments, Heidelburg, Germany). After incubation in ACSF at 34°C for at least 1 hour, a single brain slice was transferred to the recording chamber and perfused continuously with oxygenated ACSF (31 ± 1°C) at a flow rate of about 2 mL·min^{-1}.

2.9.2. Field potential recording and long-term potentiation induction

The CeA represents the major output nucleus of the amygdala and processes information from other amygdala nuclei and from

widespread brain areas. In this study, 2 lines of input to the CeA including the PB–CeA synapse and the BLA-CeA synapse were examined, in which the PB-CeA synapse provides nociceptive inputs from the spinal cord and brainstem,[5,30] whereas the BLA-CeA synapse transmits highly integrated polymodal sensory information (including stress and nociceptive input) from thalamic and cortical areas.[62,70,78] Field excitatory postsynaptic potentials (fEPSPs) at the BLA-CeA synapse and the PB-CeA synapse were evoked by a single-pulse test stimulation (0.1-1.0 mA, 0.1-ms duration, delivered at 1-minute intervals) of either the BLA or the PB, using a concentric bipolar stimulating electrode (Friedrick Haer & Co) supplied by a stimulator (STG4002; Multi-Channel Systems, Reutlingen, Germany). The fEPSPs were recorded in the CeA with glass Ag/AgCl microelectrodes filled with ACSF (tip resistance 1-3 MΩ). For long-term potentiation (LTP) induction, fEPSP amplitudes were adjusted to ~30% of maximal response and recorded for 20 minutes (under test stimulation) as baseline. Then, a conditioning HFS consisting of 3 trains of tetanus stimuli (1.5× rheobase, 100 Hz for 1 second, at 20-second intervals) was delivered to either the BLA or the PB for the induction of LTP at the BLA-CeA synapse and the PB-CeA synapse, respectively. Subsequent fEPSPs were recorded for additional 60 minutes under the same test stimulation as used for baseline, and their amplitudes were normalized to baseline values. Bicuculline (10 μM) was present in the perfusion solution to block inhibitory synaptic transmission.

2.10. Histology

At the end of each experiment in the in vivo electrophysiology, the stimulating and recording sites in EC, BLA, or CeA were marked by injecting DC current (250 μA for 3 minutes) through the respective stimulating and recording electrode. The brain was removed and submerged in 10% formalin and potassium ferrocyanide. Tissues were stored in 20% sucrose before they were frozen sectioned at 30 μm. Sections were stained with Neutral Red, mounted on gel-coated slide, and covered with a cover slip. The boundaries of EC and the different amygdala nuclei were easily identified under the microscope. Stimulating/recording sites were verified histologically and plotted on standard diagrams adapted from Paxinos and Watson [77] (supplementary data: Figs. S1A–D, available online at http://links.lww.com/PAIN/A378). Animals with wrongly placed electrode were excluded from analysis.

To verify the cannula placement in the intra-BLA/CeA microinjection experiments, 0.5 μL of black India ink was injected into the BLA/CeA immediately before the rat was euthanized at the end of each experiment, and the ink diffusion into the BLA/CeA was histologically evaluated. In brief, deeply anesthetized rats were perfused transcardially with 0.9% isotonic saline followed by 4% paraformaldehyde. After fixing in paraformaldehyde at 4°C overnight, the brains were transferred to a 20% sucrose solution in saline for cryoprotection. Coronal sections of 30 μm were cut on a microtome, mounted on charged slides, and stained with cresyl violet staining solution (Beyotime Biotechnology, Shanghai, China) following Nissl staining method. The sections were examined under a light microscope (Leica DMI 3000B, Wetzlar, Germany), and determination of the location of infusion needle tips within the BLA or the CeA was made according to the standardized atlas plates of Paxinos and Watson[77] by an observer blind to drug treatment condition (supplementary data: Fig. S1E and F, available online at http://links.lww.com/PAIN/A378). Animals with wrongly placed cannulae were excluded from analysis.

2.11. Western blot analysis

2.11.1. Total protein preparation

Deeply anesthetized rats were decapitated and their brains were rapidly removed. Two to three 500-μm amygdala slices from both left and right hemispheres were then sectioned and the CeA was quickly cut out, homogenized, and processed as previously described.[7,73] In brief, the CeA tissues were removed and immediately homogenized in ice-chilled lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP40 (Sigma-Aldrich), 0.5% sodium deoxycholate (Sigma-Aldrich), 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail (Roche, Indianapolis, IN). Then, the homogenates were centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatant was analyzed. The concentration of protein was measured with a BCA assay kit (Pierce), and an equal amount of protein samples was denatured and then analyzed by Western blotting.

2.11.2. Synaptosomal fraction preparation

Synaptosomal fraction was extracted using modified methods as described in previous studies.[26,27] Briefly, the CeA tissues were homogenized in ice-chilled lysis buffer containing 320 mM sucrose, 10 mM HEPES, 2 mM EDTA, and 1 mM PMSF. The extract was centrifuged at 1000*g* for 10 minutes to remove nuclei and large debris (P1). The supernatant (S1) was centrifuged at 10,000*g* for 15 minutes to obtain the synaptosomal fraction (P2). The P2 pellet were solubilized with 1% SDS in Tobacco Etch Virus Protease (TEVP) buffer containing 10 mM Tris–HCl (pH 7.4), 5 mM NaF, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 2 mM PMSF, and 1% SDS at 4°C for 1 hour and centrifuged at 15,000*g* for 5 minutes to get the supernatant. The concentration of protein was determined using a BCA assay kit (Pierce, Rockford, IL).

2.11.3. Western blotting

Equal amount of protein samples (30 μg) were denatured and then separated through SDS–polyacrylamide gel electrophoresis using 8% running gels and transferred to a polyvinylidene difluoride filters membrane (Bio-Rad, Hercules, CA). After blocking with 5% nonfat milk in Tris-buffered saline and Tween (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 60 minutes at room temperature, the membrane was respectively incubated with the following primary antibodies at 4°C overnight: rabbit anti-rat GluN1 (1:1000; Santa Cruz Biotechnology), rabbit anti-rat GluN2A (1:1000; Cell signaling Technology), mouse anti-rat GluN2B (1:1000; Cell signaling Technology), mouse anti-GAPDH (1:1000; Santa Cruz Biotechnology), and mouse anti-β-actin (1:2000; Santa Cruz Biotechnology).

The blots were washed in Tris-buffered saline and Tween and then were incubated in horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse IgG secondary antibody (Santa Cruz Biotechnology). Protein bands were visualized using an enhanced chemiluminescence detection kit (Pierce) followed by autoradiography using Hyperfilm MP (Santa Cruz Biotechnology). The standardized ratio of GluN1, GluN2A, or GluN2B synaptic protein to GAPDH band density, or total protein to β-actin band density, was used to calculate the alteration of corresponding protein expression.

2.12. Experimental design

2.12.1. Experiment 1

The aim of this experiment was to investigate the role of chronic FS stress on nerve injury–induced pain hypersensitivity in SNI rats.

Adult male rats (n = 78) were randomly assigned to 7 groups, including CFSS group, sham FS group, SNI group, sham SNI group, CFSS + SNI group, sham FS + SNI group, and CFSS + sham SNI group, each group of 8 to 14, used for investigating effects of CFSS on depressive- and anxiety-like behaviors and on nerve injury–induced mechanical allodynia (**Fig. 1**).

In CFSS group and sham FS group, animals were exposed to FS or sham FS once per day in 15-minute sessions for 14 consecutive days. Alterations of body weight gains during days 1 to 5, 5 to 10, and 10 to 15 stress exposures, combined with sucrose preference test, forced swim test (FST), and EPM test on day 15 post-FS exposure, were then performed to evaluate the stress-induced depressive- and anxiety-like behaviors, respectively (**Fig. 1A–F**). Meanwhile, the ipsilateral PWT was performed on days 1, 3, 7, 10, and 14 after FS stress (**Fig. 1G**).

In SNI group and sham SNI group, animals were first fed parallelly along with FS or sham FS rats and ipsilateral PWT was respectively assessed on day 14 and day 0 (before SNI surgery) as baseline. Then, SNI or sham SNI surgery was carried out on the left thigh of rat, and the ipsilateral PWT was measured on days 1, 3, 7, 10, and 14 after surgery (**Fig. 1G**).

In CFSS + SNI group, sham FS + SNI group, and CFSS + sham SNI group, animals were first exposed to FS or sham FS conditioning stress for 14 consecutive days (once per day in 15-minute sessions) and then an SNI or a sham SNI surgery was performed on the left thigh. The ipsilateral PWT was assessed on day 14 and day 0 (before surgery), and on days 1, 3, 7, 10, and 14 after surgery, respectively (**Fig. 1G**). At the end of each experiment, inclined-plate test was performed to assess the animal's locomotor function in all the above groups (**Fig. 1H**). A timeline schematic illustrating the experimental procedure was shown in **Figure 1I**.

2.12.2. Experiment 2

The aim of this experiment was to explore the role for CFSS on both neuronal activities in the BLA and CeA, as well as on synaptic plasticity at the BLA-CeA synapse and the PB-CeA synapse (**Figs. 2–6**). In this experiment, 304 adult male rats were divided into CFSS group, sham FS group, SNI group, sham SNI group, CFSS + SNI group, sham FS + SNI group and CFSS + sham SNI group, each group of 10 to 17, used for in vivo electrophysiological recording (**Figs. 2, 3 and 5**), and in other experiments, each group of 5 to 7, used for in vitro slice LTP recording (**Figs. 4 and 6**). All protocols like FS stress, SNI surgery, and FS conditioning sessions were conducted similarly as described in the *Experiment 1* section, and all electrophysiological recordings were performed after pain behavioral assessments during day 7 to day 10 postsurgery (**Fig. 1I**).

2.12.3. Experiment 3

In this experiment, we performed in vitro slice LTP recording and pharmacological behavioral test to determine the role for GluN2B-NMDA receptors in chronic FS-induced augmentation of LTP at the PB-CeA synapse and pain exacerbation in SNI rats (**Fig. 7**).

For in vitro slice LTP recording, 37 adult male rats (n = 5-7 per group) were divided into Ro/CFSS group, saline/CFSS group, Ro/SNI group, saline/SNI group, Ro/CFSS + SNI group, and saline/CFSS + SNI group. Likewise, all protocols like FS stress, SNI surgery, and FS conditioning sessions were conducted similarly as described in the *Experiment 1* section, and all electrophysiological recordings were performed after pain behavioral assessments during day 7 to day 10 postsurgery (**Fig. 1I**). In each group, the brain slice was pretreated with Ro 25-6981 (10 μM) or normal saline for 20 minutes of consecutive perfusion, followed by a conditioning HFS to induce LTP at the PB-CeA synapse (**Fig. 7A–C**).

For pharmacological behavioral test, 39 adult rats (n = 9-10 per group) were randomly assigned to ifenprodil/SNI group, vehicle/SNI group, ifenprodil/CFSS + SNI group, and vehicle/CFSS + SNI group. Both the SNI surgery and FS conditioning sessions were conducted according to the protocols as described in the *Experiment 1* section. Ifenprodil (1 $\mu g \cdot \mu L^{-1}$) or vehicle was administered into the CeA (0.5 μL per injection) twice per day at a 12-hour interval, for 3 consecutive days (ie, from day 4 to day 6 following SNI surgery when stable pain allodynia appeared). The ipsilateral (the injured left hind limb) PWT of rat was measured on day 0 (before SNI surgery), day 1, and on days 3 to 7 postsurgery, respectively. On those days of day 4, day 5, and day 6 when ifenprodil was applied, the pain behavior was examined 30 minutes after the second drug injection each day (**Fig. 7D and E**). To verify the cannula placement, 0.5 μL of black India ink was injected into the CeA immediately before the rat was euthanized at the end of each experiment, and ink diffusion into the CeA was histologically evaluated (supplementary data: Fig. S1F, available online at http://links.lww.com/PAIN/A378). Animals with wrongly placed cannulae were excluded from analysis.

2.12.4. Experiment 4

In this experiment, we performed Western blotting to examine the synaptic protein expression of GluN2B-NMDA receptors in synaptosomal fraction extracted from the right CeA tissue, as well as the total protein expression of the receptors in both left and right CeA. Twenty-eight adult rats (n = 4 per group) were divided into CFSS group, sham FS group, SNI group, sham SNI group, CFSS + SNI group, sham FS + SNI group, and CFSS + sham SNI group, used for expression of receptors in synaptosomal fraction (**Fig. 8**). In other experiments, 16 rats (n = 4 per group) were assigned to naive group, CFSS group, SNI group, and CFSS + SNI group, used for total protein expression (supplementary data: Fig. S2, available online at http://links.lww.com/PAIN/A378). All protocols like FS stress, SNI surgery, and FS conditioning sessions were conducted similarly as described in the *Experiment 1* section, and the Western blotting was conducted after pain behavioral assessments on day 1 poststress or on day 7 postsurgery for respective CFSS and SNI/CFSS + SNI treatment (**Fig. 1I**).

2.12.5. Experiment 5

The aim of this experiment was to determine whether the stress-related depression underlies the chronic FS-induced exacerbation of neuropathic pain. First, we investigated effects of antidepressants including imipramine and ifenprodil on CFSS-induced depressive-like behaviors and the exacerbation of SNI-elicited pain allodynia (**Fig. 9**). Imipramine is a well-established tricyclic antidepressant that has been used clinically for many years.[95,108,110] Similar as the classic antidepressant imipramine, ifenprodil used chronically at a low dose (3 $mg \cdot kg^{-1}$) by intraperitoneal administration also produces a significant antidepressant-like effect in the FST and therefore to be used here mainly as an antidepressant but not merely as a GluN2B-NMDA receptor antagonist.[32,33] In this part of the experiment, 68 adult rats (n = 11-12 per group) were randomly assigned to imipramine/CFSS group, ifenprodil/CFSS, saline/CFSS group, as well as imipramine/CFSS + SNI group, ifenprodil/CFSS + SNI group, and saline/CFSS + SNI group. According to previous

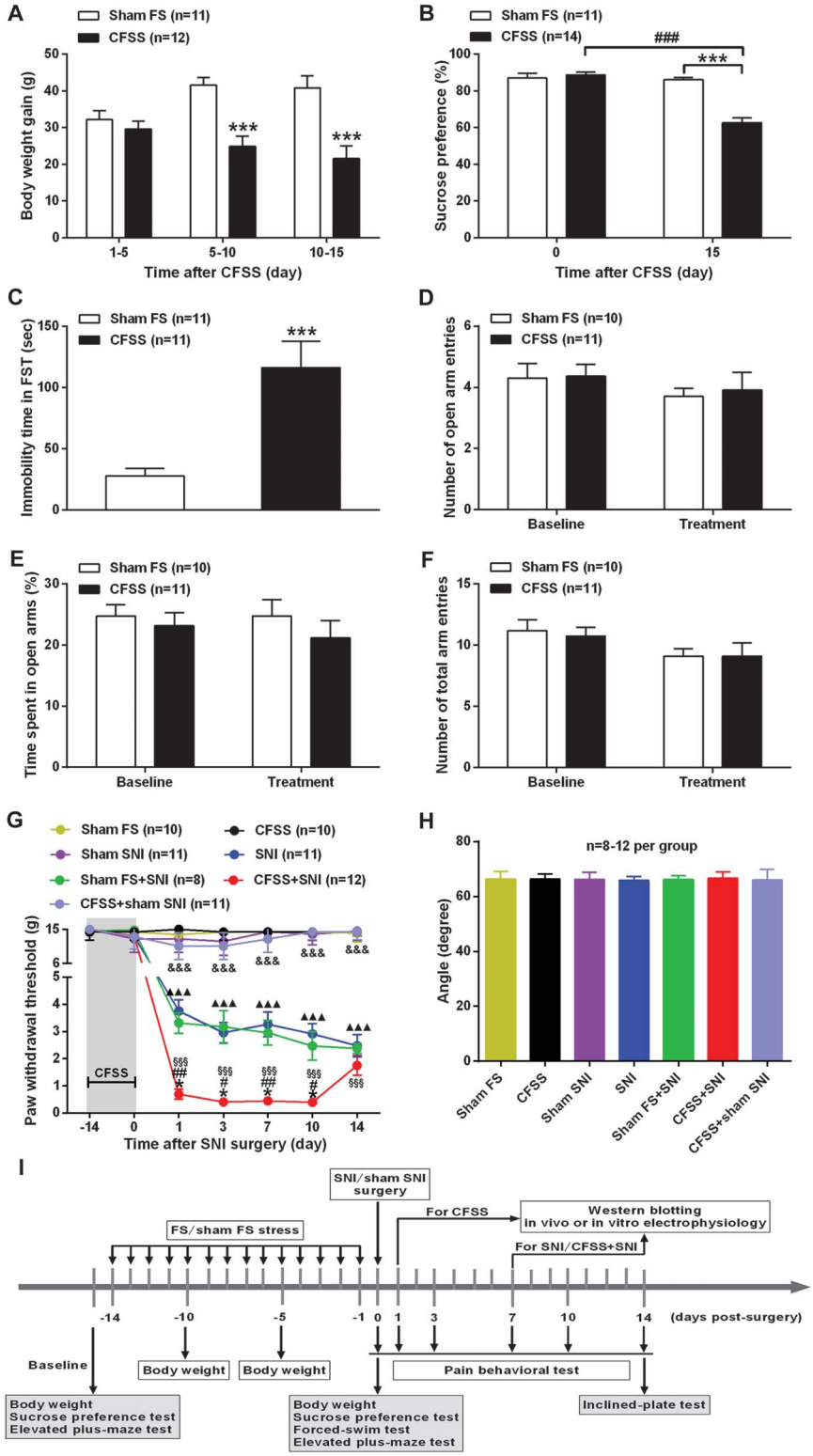

Figure 1. Effects of chronic forced swim stress (CFSS) on anxiety- and depressive-like behaviors as well as on spared nerve injury (SNI)-induced neuropathic pain in rats. (A–C) Assessment of depressive-like behaviors. Note that chronic exposure of forced swimming (FS) to rats results in a significant depressive-like behavior as indicated by a decreased body weight gains (A), a reduced preference for sucrose consumption (B), and an increased immobility time during the forced swim tests (C). ***$P < 0.001$, in contrast to sham FS rats; ###$P < 0.001$, in contrast to day 0 (baseline), 2-way analysis of variance (ANOVA) or 2-tailed unpaired *t* test, $n =$ 10 to 14 per group. (D–F) Assessment of anxiety-like behaviors. Note that CFSS cannot induce anxiety-like behavior in rats, as assessed by the elevated plus-maze test. $P > 0.05$, 2-way ANOVA, $n = 11$ CFSS and 10 sham FS. (G) Assessment of mechanical allodynia. Note that while SNI surgery produces a decreased ipsilateral paw withdrawal threshold (PWT) in nonstressed animals, pre-exposure of CFSS to SNI surgery results in a more reduction of PWT in stress-treated SNI rats. ▲▲▲$P < 0.001$, SNI versus sham SNI; *$P < 0.05$, CFSS + SNI versus sham FS + SNI; #$P < 0.05$, ##$P < 0.01$, CFSS + SNI versus SNI; §§§$P < 0.001$, CFSS + SNI versus CFSS; &&&$P < 0.001$, CFSS + SNI versus CFSS + sham SNI, 2-way ANOVA, $n = 8$ to 12 per group. (H) Assessment of locomotor function. No significant motor dysfunction is found in rats subjected to CFSS, SNI surgery, or CFSS plus SNI surgery, as assessed by inclined-plate test. $P > 0.05$, 1-way ANOVA, $n = 8$ to 12 per group. (I) Schematic representation of the experimental procedure.

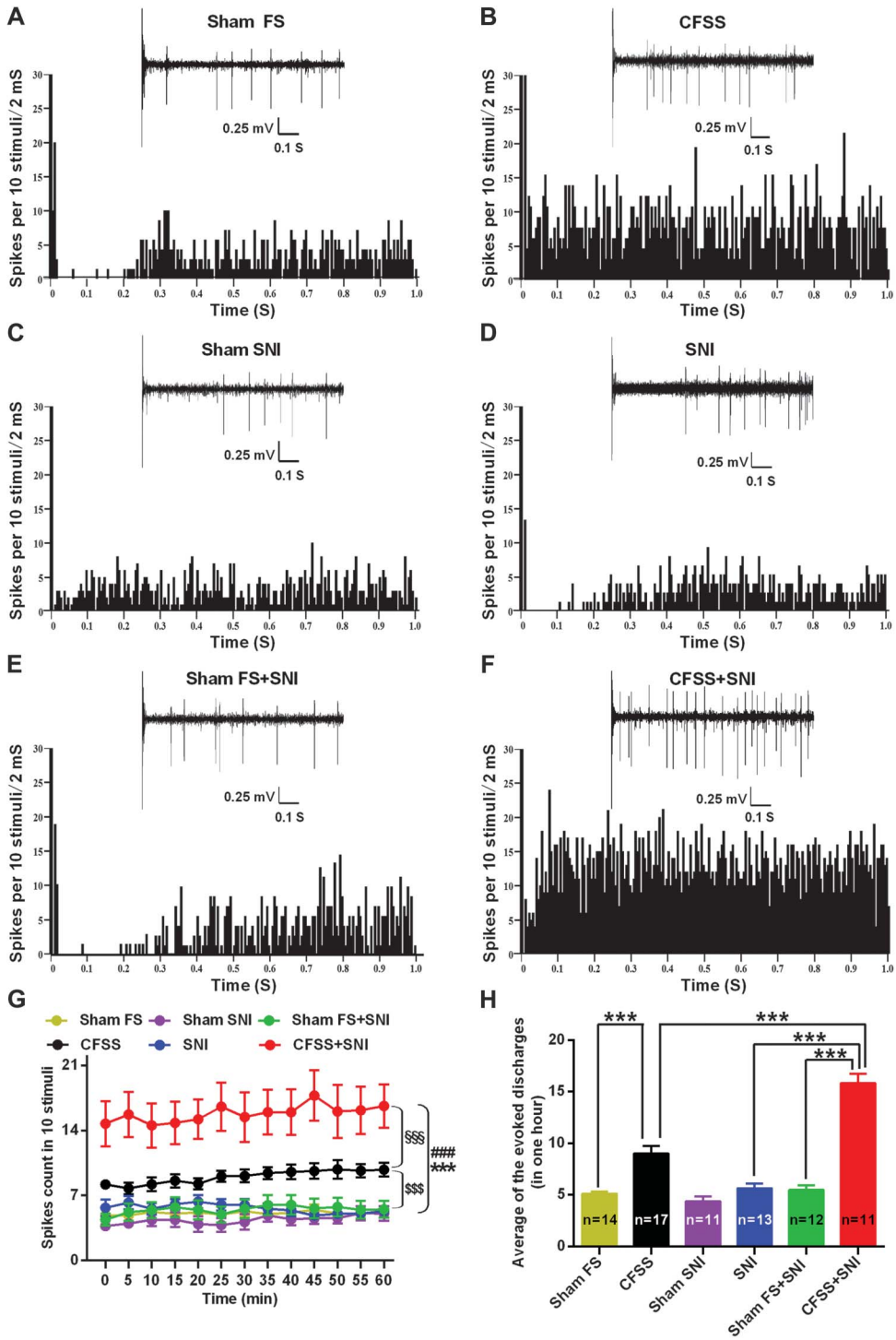

Figure 2. Effects of chronic forced swimming stress (CFSS) on electrically evoked responses of basolateral amygdala (BLA) neurons by the stimulation of external capsule (EC). (A–F) Representative electrically evoked responses of BLA neurons in sham forced swimming (FS) (A), CFSS (B), sham spared nerve injury (SNI) (C), SNI (D), sham FS + SNI (E), and CFSS + SNI (F) rats, respectively. Panels illustrate the poststimulus histogram of the electrically evoked neuronal responses. Inset shows the original recordings of the first electrically evoked neuronal responses. Scale bar: 0.25 mV, 0.1 second. (G) Total spike count of the electrically evoked neuronal responses in 10 stimuli. $$$$P < 0.001$, CFSS versus sham FS; ***$P < 0.001$, CFSS + SNI versus sham FS + SNI; §§§$P < 0.001$, CFSS + SNI versus CFSS; ###$P < 0.001$, CFSS + SNI versus SNI, 1-way analysis of variance, $n = 11$ to 17 per group. (H) Average of the evoked discharges in 60 minutes. Note that the average evoked responses of BLA neurons during 1 hour are statistically increased in the CFSS-treated rats as compared with the sham FS rats. Pre-exposure of chronic FS to SNI rats results in a more significant increase in evoked discharges of BLA neurons in the CFSS + SNI rats as compared with the sham FS + SNI rats, the CFSS rats, and the SNI rats, respectively. ***$P < 0.001$, 1-way analysis of variance, $n = 11$ to 17 per group.

reports,[32,33,54] either imipramine (10 mg·kg^{-1}) or ifenprodil (3 mg·kg^{-1}) was administered to rats for 14 consecutive days concomitantly with chronic FS conditioning. The drugs were i.p. delivered to rats once per day at 30 minutes before the FS stress training. All protocols like FS stress, SNI surgery, FS conditioning sessions, as well as the assessment on depressive-like behaviors and the animal's locomotor function were conducted similarly as described in the *Experiment 1* section. Alterations of body weight

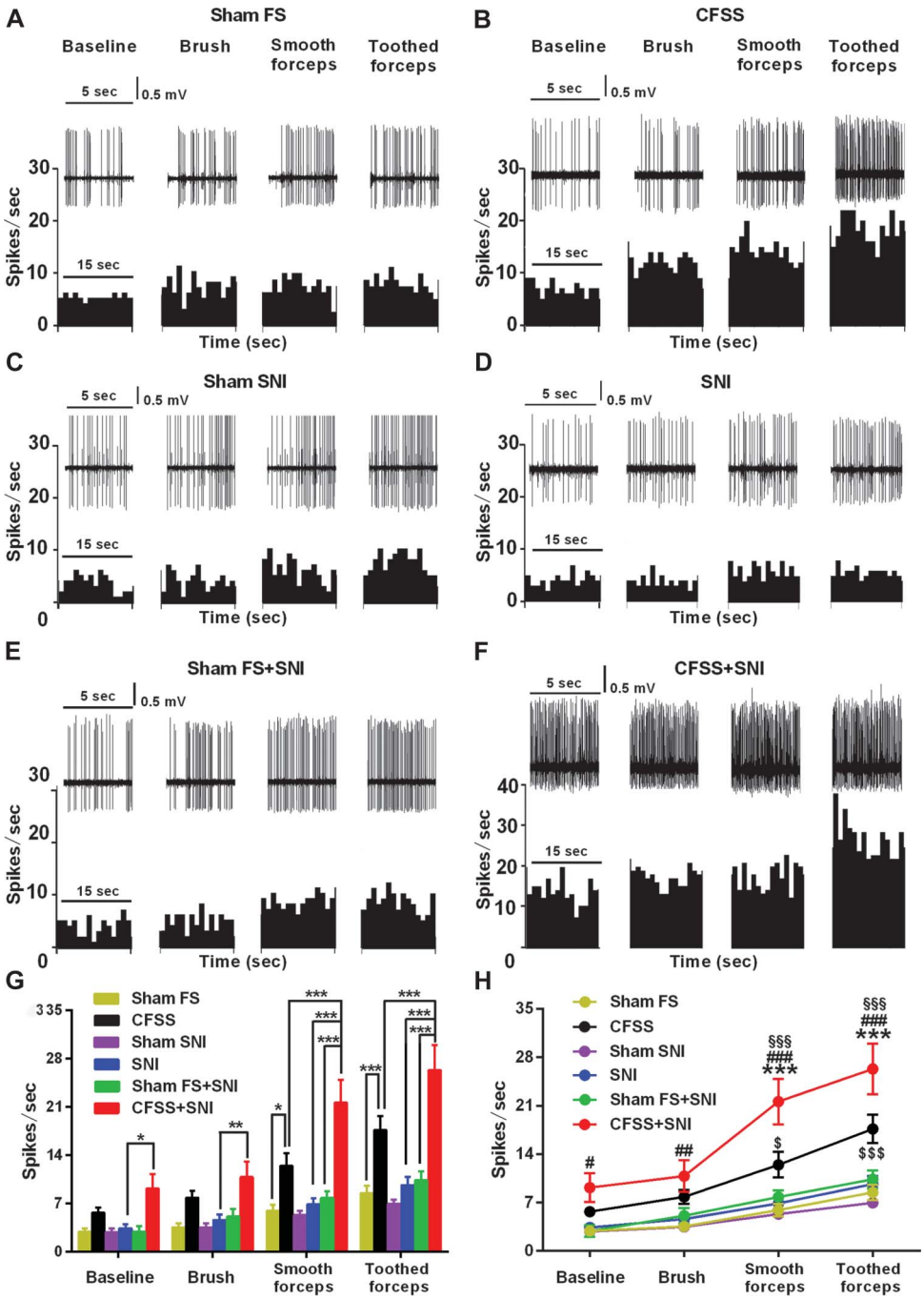

Figure 3. Effects of chronic forced swimming stress (CFSS) on spontaneous activity of basolateral amygdala (BLA) projection neurons. (A–F) Representative background activities and evoked responses of BLA neurons to brief (15 seconds) innocuous (brush) and noxious (smooth forceps and toothed forceps) mechanical stimuli in sham forced swimming (FS) (A), CFSS (B), sham spared nerve injury (SNI) (C), SNI (D), sham FS + SNI (E), and CFSS + SNI (F) rats, respectively. Histograms display the number of action potentials (spikes) per second (bin width, 1 second). Inset shows the original recordings of the background activities and the stimulus-evoked responses. Scale bar: 0.5 mV, 5 seconds. (G and H) Averaged background activities and evoked responses of BLA neurons (spikes per second) in different groups. (G) Bar graph. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, 2-way analysis of variance. (H) Line graph. Note that the averaged stimulus-evoked responses of BLA neurons to innocuous and noxious mechanical stimuli are statistically increased in the CFSS-treated rats as compared with the sham FS rats. Pre-exposure of chronic FS to SNI rats results in a more significant increase in the averaged stimulus-evoked responses of BLA neurons in the CFSS + SNI rats as compared with the sham FS + SNI rats, the CFSS rats, and the SNI rats, respectively. $\$P < 0.05$, $\$\$\$P < 0.001$, CFSS versus sham FS; $^{***}P < 0.001$, CFSS + SNI versus sham FS + SNI; $\S\S\S P < 0.001$, CFSS + SNI versus CFSS; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$, CFSS + SNI versus SNI, 2-way analysis of variance, $n = 17$ to 24 per group.

gains during days 1 to 5, 5 to 10, and 10 to 15 stress exposures, combined with sucrose preference test and FST on day 15 post-FS exposure, were then performed to evaluate the stress-induced depressive-like behaviors (**Fig. 9A–D**). The SNI surgery was performed at the end of CFSS training, and the ipsilateral PWT was performed on days 1, 3, 7, 10, and 14 after FS stress (**Fig. 9E**). At the end of each experiment, inclined-plate test was performed to assess the animal's locomotor function in all the above groups (**Fig. 9D**). A timeline schematic illustrating the experimental procedure is shown in **Figure 9F**.

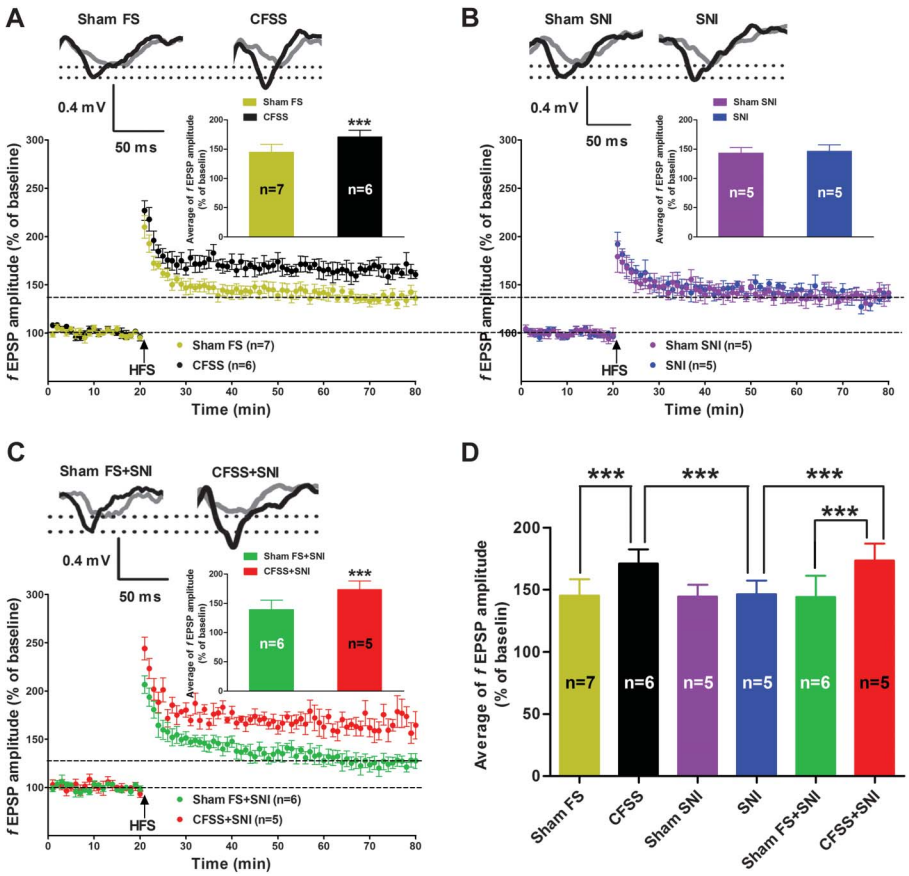

Figure 4. Effects of chronic forced swimming (FS) stress on the long-term potentiation (LTP) of field excitatory postsynaptic potentials (fEPSPs) at the basolateral amygdala (BLA)–central nucleus of the amygdala (CeA) synapse. The fEPSPs are evoked by a test stimulation (0.1-1.0 mA, 0.1-ms duration, delivered at 1-minute intervals) of the BLA using a concentric bipolar stimulating electrode. (A–C) Histograms display the mean time course of fEPSPs before and after high-frequency stimulation (HFS) (0.1-ms duration, 1.5× rheobase, 100 Hz, 300 pulses given in 3 trains of 1-second duration at 20-second intervals; arrow). Traces at top are representative fEPSPs recorded at 10 minutes before and 60 minutes after HFS of the BLA. Scale bar: 0.4 mV, 50 milliseconds. The inserted bar graph indicates the mean amplitude of fEPSPs during the 20 to 80 minutes following HFS. ***$P < 0.001$, 2-tailed unpaired *t* test. (D) Statistical analysis of mean fEPSPs amplitude during the 20 to 80 minutes following HFS of the BLA in rats obtained from the above groups. Note that chronic exposure of FS to intact rats induces a notable augmentation of LTP at the BLA-CeA synapse. Pre-exposure of spared nerve injury (SNI) rats to chronic FS results in a more significant increase in the LTP of fEPSPs at the BLA-CeA synapse. ***$P < 0.001$, 1-way analysis of variance, $n = 5$ to 7 per group.

Second, to determine whether the analgesic effects of the antidepressants are specific to the CFSS-induced allodynia and whether the commonly used analgesic has an efficacy to the CFSS-induced exacerbation of chronic pain, we performed additional 2 experiments to examine: (1) effects of the antidepressants imipramine or ifenprodil on pain behaviors in SNI rats and (2) effects of indomethacin (Indo), a nonsteriodal anti-inflammatory drugs (NSAIDs), on CFSS-induced exacerbation of pain allodynia in stress-treated SNI (CFSS + SNI) rats (supplementary data: Fig. S3, available online at http://links.lww.com/PAIN/A378). In this part of the experiment, 85 adult rats (n = 7-13 per group) were randomly assigned to imipramine/SNI group, ifenprodil/SNI group, vehicle/SNI group, as well as Indo (5 mg·kg^{-1})/CFSS + SNI group, Indo (10 mg·kg^{-1})/CFSS + SNI group, and vehicle/CFSS + SNI group for pretreatment, and Indo (5 mg·kg^{-1})/CFSS + SNI group, Indo (10 mg·kg^{-1})/CFSS + SNI group, and vehicle/CFSS + SNI group for posttreatment. In SNI rats, imipramine (10 mg·kg^{-1}) or ifenprodil (3 mg·kg^{-1}) was i.p. administered at 30 minutes before SNI surgery and then repeated once per day for 7 consecutive days after SNI surgery. The ipsilateral PWT was assessed on day 0 (before surgery) and on days 1, 3, 7, 10, and 14 after surgery, respectively (Fig. S3A, http://links.lww.com/PAIN/A378). In CFSS + SNI rats, both low dose (5 mg·kg^{-1}) and high dose (10 mg·kg^{-1}) of indomethacin were i.p. administered to stress-treated SNI rats by 2 ways, ie, pretreatment and posttreatment. In pretreatment experiments, Indo (5 or 10 mg·kg^{-1}) was i.p. applied once per day at 30 minutes before FS stress training, concomitantly with 14 consecutive days for chronic FS preconditioning. Then SNI surgery was performed at the end of CFSS training, and the ipsilateral PWT was evaluated on day 14 and day 0 (before surgery) and on days 1, 3, 7, 10, and 14 after surgery, respectively (Fig. S3B, http://links.lww.com/PAIN/A378). In posttreatment experiments, rats were first exposed to 14 consecutive days of chronic FS conditioning, then Indo (5 or 10 mg·kg^{-1}) was i.p. administered to rats at 30 minutes before SNI surgery, and repeated once per day for 7 consecutive days after SNI surgery. The ipsilateral PWT was assessed on day 0 (before surgery) and on days 1, 3, 7, 10, and 14 after surgery, respectively (Fig. S3C, http://links.lww.com/PAIN/A378). At the end of each experiment, inclined-plate test was performed to assess the animal's locomotor function in all the above groups (Fig. S3D, http://links.lww.com/PAIN/A378). A timeline schematic illustrating the experimental procedure of Figure S3A–C (http://links.lww.com/PAIN/A378) was shown in Figure S3E–G (http://links.lww.com/PAIN/A378), respectively.

Moreover, we conducted another additional experiment to determine whether intra-BLA administration of the antidepressants

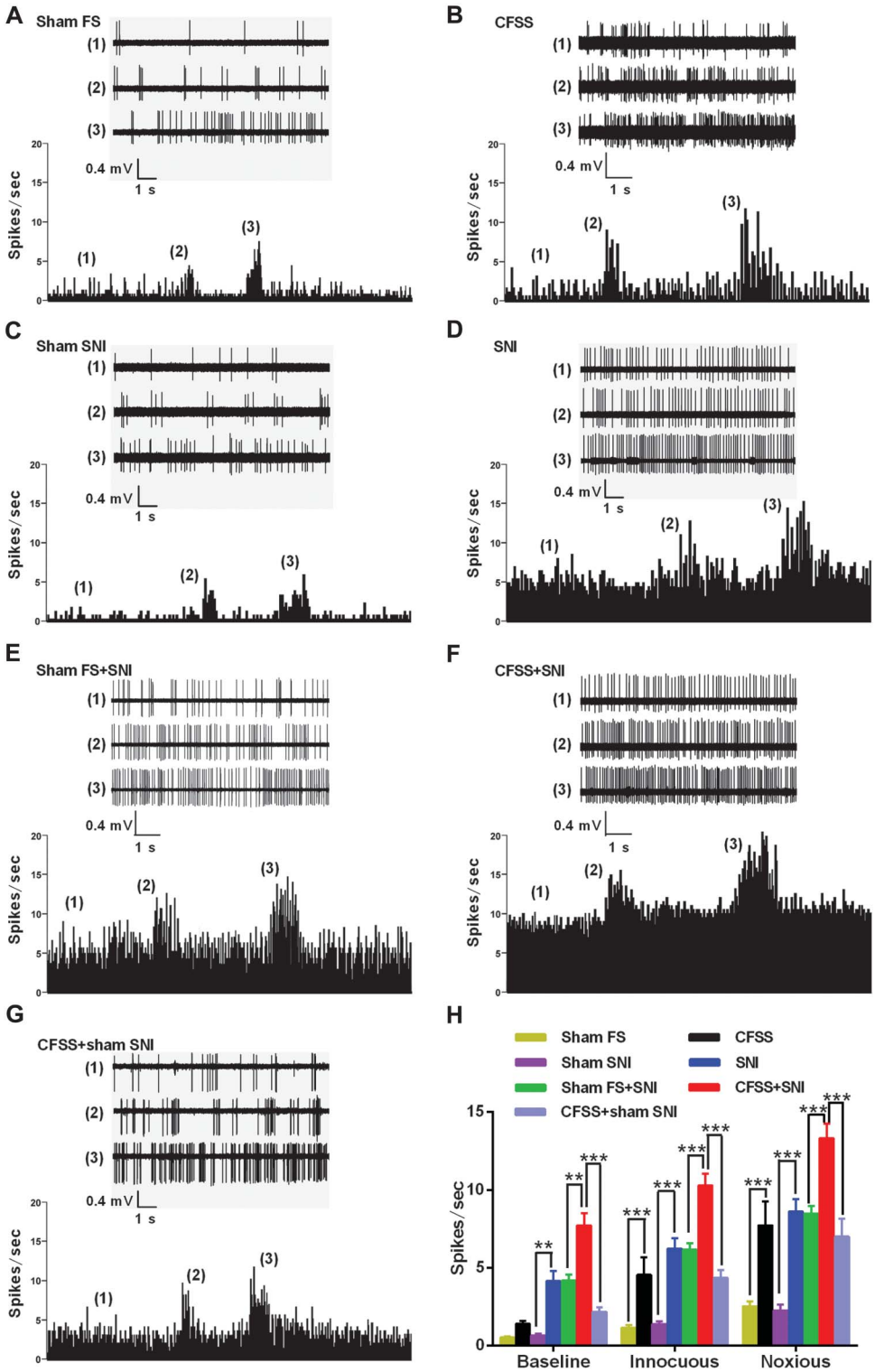

Figure 5. Effects of chronic forced swim stress (CFSS) on spontaneous activity of multireceptive (MR) neurons in the central nucleus of the amygdala (CeA). (A–G) Representative background activities (1) and evoked responses of MR neurons to brief (15 seconds) innocuous (brush) (2) and noxious (pinch) (3) mechanical stimuli in sham forced swimming (FS) (A), CFSS (B), sham spared nerve injury (SNI) (C), SNI (D), sham FS + SNI (E), CFSS + SNI (F), and CFSS + sham SNI (G) rats, respectively. Histograms display the number of action potentials (spikes) per second (bin width, 1 second). Inset shows the original recordings of the background activities and the stimulus-evoked responses of MR neurons to brief (15 seconds) innocuous (brush) (2) and noxious (pinch) (3) mechanical stimuli. Scale bar: 0.4 mV, 1 second. (H) Averaged background activities and evoked responses of MR neurons (spikes per second) in different groups. Note that the averaged stimulus-evoked responses of MR neurons to innocuous and noxious mechanical stimuli are statistically increased both in the CFSS-treated rats and in the SNI rats. Pre-exposure of chronic FS to SNI rats results in a more significant increase in the averaged stimulus-evoked responses of MR neurons in the CFSS + SNI rats as compared with the sham FS + SNI rats and the CFSS + sham SNI rats. $^{**}P < 0.01$, $^{***}P < 0.001$, 2-way analysis of variance, $n = 10$ to 15 per group.

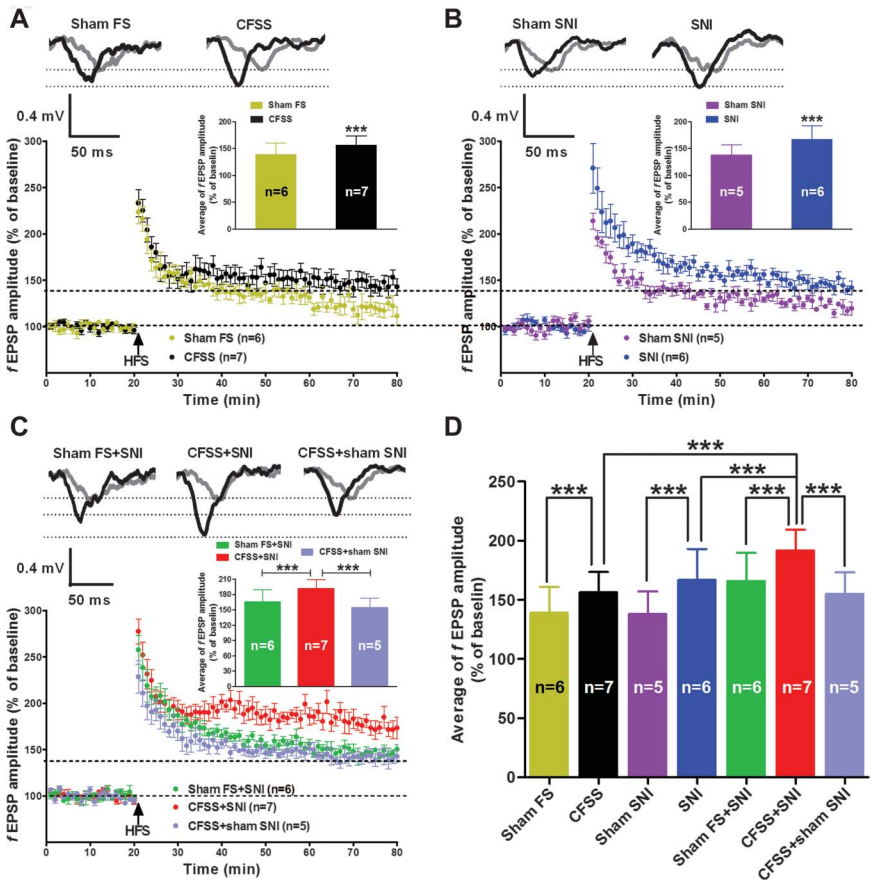

Figure 6. Effects of chronic forced swimming (FS) stress on the long-term potentiation (LTP) of field excitatory postsynaptic potentials (fEPSPs) at the parabrachial-central nucleus of the amygdala (PB-CeA) synapse. The fEPSPs are evoked by a test stimulation (0.1-1.0 mA, 0.1-ms duration, delivered at 1-minute intervals) of the PB using a concentric bipolar stimulating electrode. (A–C) Histograms display the mean time course of fEPSPs before and after high-frequency stimulation (HFS) (0.1-ms duration, 1.5× rheobase, 100 Hz, 300 pulses given in 3 trains of 1-second duration at 20-second intervals; arrow). Traces at top are representative fEPSPs recorded at 10 minutes before and 60 minutes after HFS of the PB. Scale bar: 0.4 mV, 50 milliseconds. The inserted bar graph indicates the mean amplitude of fEPSPs during the 20 to 80 minutes following HFS. ***$P < 0.001$, 2-tailed unpaired *t* test or 1-way analysis of variance. (D) Statistical analysis of mean fEPSPs amplitude during the 20 to 80 minutes following HFS of the PB in rats obtained from the above groups. Note that both chronic FS and spared nerve injury (SNI) surgery enhances the LTP of fEPSPs at the PB-CeA synapse. Pre-exposure of chronic FS to SNI rats produces a more significant increase in the LTP of fEPSPs at the PB-CeA synapse. ***$P < 0.001$, 1-way analysis of variance, $n = 5$ to 7 per group.

also has effects on chronic FS-induced depressive-like behaviors in stressed rats and on chronic FS-induced exacerbation of pain allodynia in stress-treated SNI rats (supplementary data: Fig. S4, available online at http://links.lww.com/PAIN/A378). Sixty adult rats (n = 9-11 per group) were randomly assigned to imipramine/CFSS group, ifenprodil/CFSS group, saline/CFSS group, as well as imipramine/CFSS + SNI group, ifenprodil/CFSS + SNI group, and saline/CFSS + SNI group. Imipramine (30 $\mu g \cdot \mu L^{-1}$) or ifenprodil (10 $\mu g \cdot \mu L^{-1}$) was administered into the BLA (0.5 μL per injection) for 14 consecutive days concomitantly with chronic FS conditioning. The drugs were delivered to rats once per day at 30 minutes before the FS conditioning. All protocols like FS stress, SNI surgery, FS conditioning sessions, and the assessment on depressive-like behaviors and the animal's locomotor function were conducted similarly as described in the *Experiment 1* section. Alterations of body weight gains during days 1 to 5, 5 to 10, and 10 to 15 stress exposures, combined with sucrose preference test and FST on day 15 post-FS exposure, were then performed to evaluate the stress-induced depressive-like behaviors (Fig. S4A–D, http://links.lww.com/PAIN/A378). The SNI surgery was performed at the end of CFSS training, and the ipsilateral PWT was assessed on day 14 and day 0 (before surgery) and on days 1, 3, 7, 10, and 14 after surgery, respectively (Fig. S4E and F, http://links.lww.com/PAIN/A378). To verify the cannula placement, 0.5 μL of black India ink was injected into the BLA immediately before the rat was euthanized at the end of each experiment, and ink diffusion into the BLA was histologically evaluated (supplementary data: Fig. S1E, available online at http://links.lww.com/PAIN/A378). Animals with wrongly placed cannulae were excluded from analysis. At the end of each experiment, inclined-plate test was performed to assess the animal's locomotor function in all the above groups (Fig. S4D, http://links.lww.com/PAIN/A378).

2.13. Statistical analysis

Statistical analyses were performed with GraphPad Prism 6 for Windows (GraphPad Software, Inc, La Jolla, CA). All data were expressed as mean ± standard error of the mean. A 2-tailed unpaired *t* test was used for the comparison of the mean values between 2 groups. One-way analysis of variance (ANOVA) followed by Dunnett multiple comparison test or 2-way ANOVA followed by the Bonferroni post hoc test was used for multiple comparison. Differences with $P < 0.05$ were considered statistically significant.

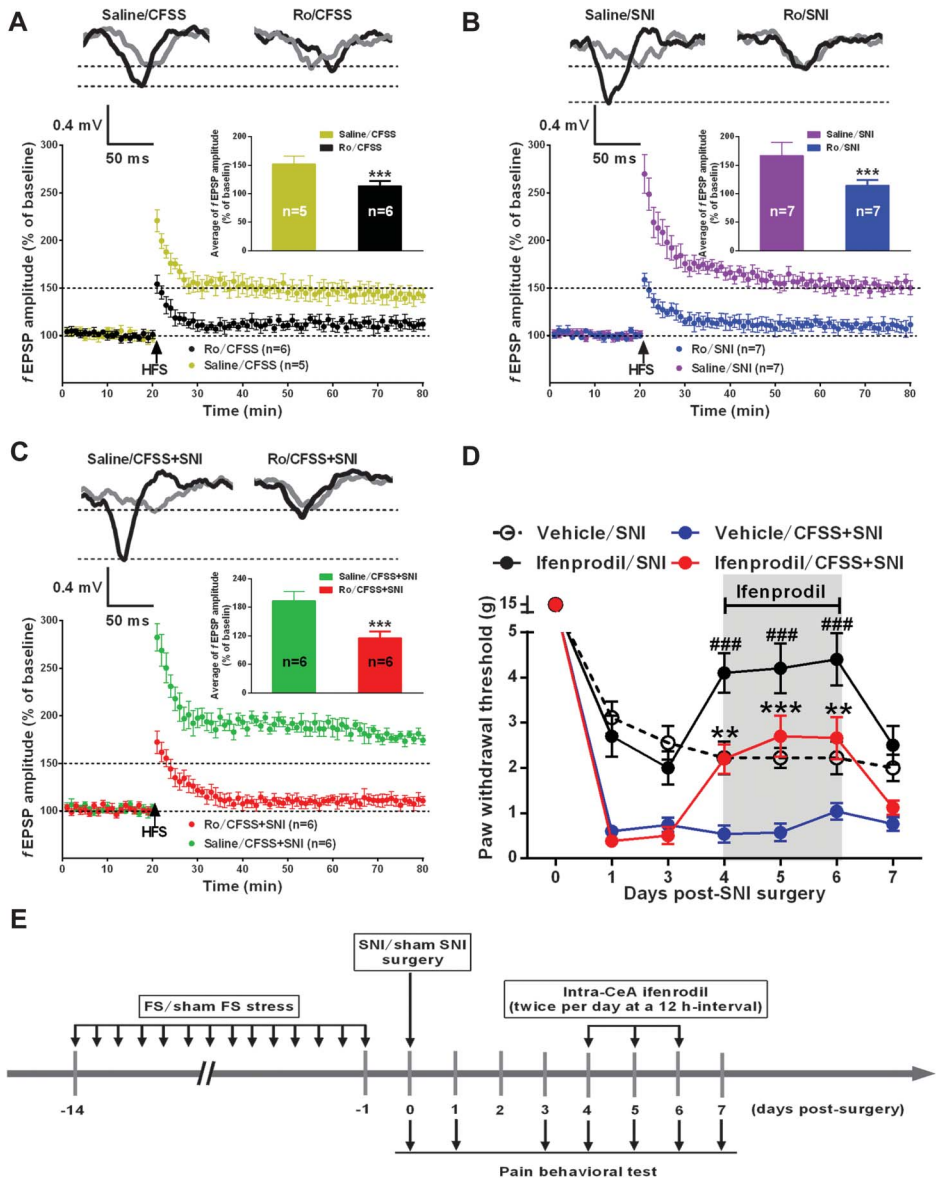

Figure 7. Effects of GluN2B-NMDA receptor antagonist on both chronic forced swimming (FS)-induced augmentation of long-term potentiation (LTP) at the parabrachial-central nucleus of the amygdala (PB-CeA) synapse and the exacerbation of pain allodynia in spared nerve injury (SNI) rats. (A–C) Effects of GluN2B-NMDA receptor antagonist Ro 25-6981 on the LTP of field excitatory postsynaptic potentials (fEPSPs) at the PB-CeA synapse in CFSS, SNI and CFSS+SNI rats, respectively. The fEPSPs are evoked by a test stimulation (0.1-1.0 mA, 0.1-ms duration, delivered at 1-minute intervals) of the PB using a concentric bipolar stimulating electrode. Histograms display the mean time course of fEPSPs before and after high-frequency stimulation (HFS) (0.1-ms duration, 1.5× rheobase, 100 Hz, 300 pulses given in 3 trains of 1-second duration at 20-second intervals; arrow). Traces at top are representative fEPSPs recorded at 10 minutes before and 60 minutes after HFS of the PB. Scale bar: 0.4 mV, 50 milliseconds. The inserted bar graph indicates the mean amplitude of fEPSPs during the 20 to 80 minutes following HFS. Note that Ro 25-6981 not only inhibits the CFSS- and the SNI-induced LTP at the PB-CeA synapse but also blocks the CFSS-induced augmentation of LTP at the PB-CeA synapse in SNI rats. ***$P < 0.001$, 2-tailed unpaired t test, $n = 5$ to 7 per group. (D) Effects of intra-CeA administration of ifenprodil, another GluN2B-NMDA receptor antagonist, on SNI-induced mechanical allodynia in SNI and CFSS+SNI rats, respectively. Note that ifenprodil not only inhibits the SNI-induced decrease in ipsilateral paw withdrawal threshold in nonstressed animals but also blocks the CFSS-induced exacerbation of pain allodynia in SNI rats. **$P < 0.01$, ***$P < 0.001$, ifenprodil/CFSS+SNI versus saline/CFSS+SNI; ###$P < 0.001$, ifenprodil/SNI versus saline/SNI, 2-way analysis of variance, $n = 9$ to 10 per group. (E) Schematic representation of the experimental procedure.

3. Results

3.1. Chronic forced swim stress produces both depressive-like behaviors in stressed rats and the exacerbation of nerve injury–induced mechanical allodynia in stress-treated spared nerve injury rats

Exposure to chronic stressful events is known to be a risk factor for the development of several neuropsychiatric disorders, including anxiety and depression in particular.[62] In line with previous findings,[10,15,98] we indeed observed that exposure of chronic forced swim (FS), which are stressful for animals, caused a depressive-like behavior in rats, as indicated by a decreased body weight gains, a reduced sucrose preference, and an increased immobility time during the FSTs. As shown in **Figure 1**, CFSS resulted in a decrease in body weight gains during days 5 to 10 (24.92 ± 2.74 g CFSS vs 41.55 ± 2.16 g sham FS, $P < 0.001$) and days 10 to 15 (21.58 ± 3.50 g CFSS vs 40.82 ± 3.35 g sham FS, $P < 0.001$) after stress exposure (2-way ANOVA, $n = 12$ CFSS and 11 sham FS, **Fig. 1A**). Moreover, after 14 days of stress exposure, the stressed rats showed a statistical reduction in

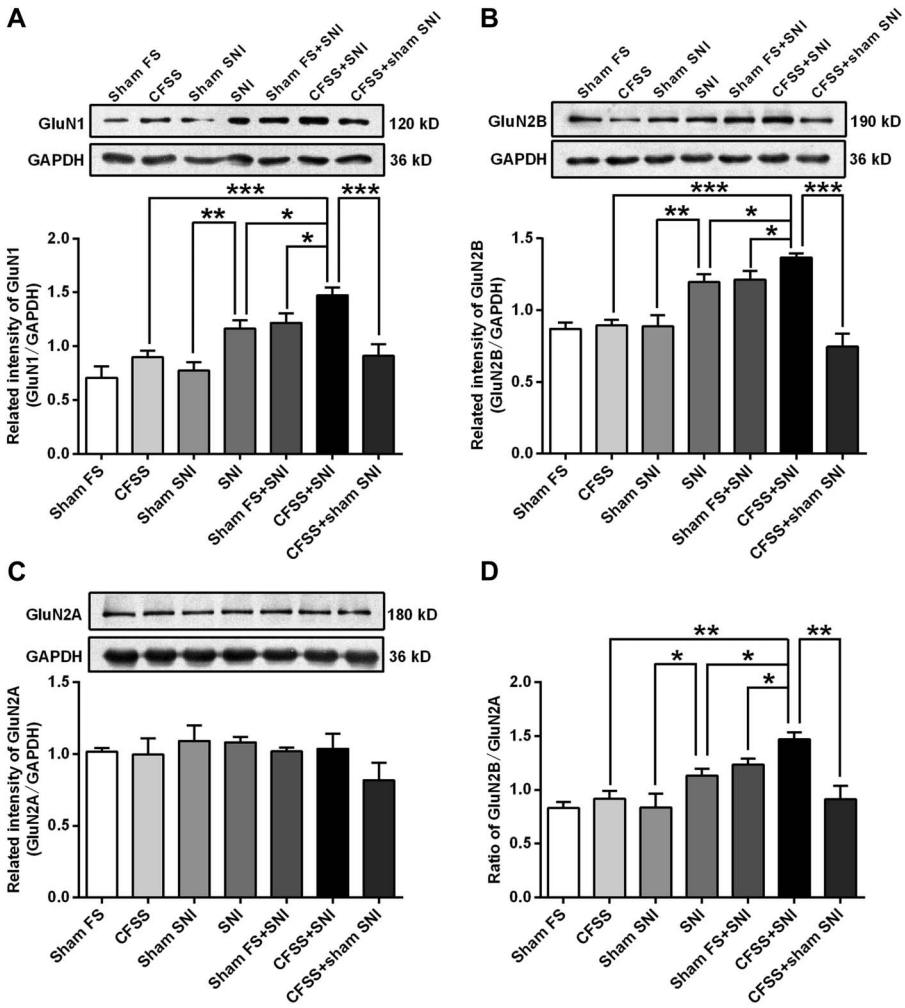

Figure 8. Effects of chronic forced swimming (FS) stress on functional expression of GluN2B-NMDA receptors in the amygdala central nucleus (CeA). (A–C) Western blot of GluN1 (A), GluN2B (B), and GluN2A (C) subunits of NMDA receptors in synaptosomal fraction extracted from the right CeA tissue. Upper: representative of Western blot bands; lower: analysis of the relative intensity of GluN1, GluN2B, and GluN2A, respectively. GAPDH is used as an internal control. (D) Ratio of GluN2B/GluN2A. Note that although chronic FS stress has no significant effect on the synaptic expression of GluN1, GluN2A and GluN2B in the CeA of intact rats, pre-exposure of spared nerve injury (SNI) rats to chronic FS indeed facilitates the SNI-induced up-regulation of GluN1, GluN2B, and the ratio of GluN2B/GluN2A in synaptosomal fraction of the CeA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, one-way analysis of variance, $n = 5$ per group.

sucrose solution intake as compared with the control animals (62.69 ± 2.65% CFSS vs 86.15 ± 1.26% sham FS, $P < 0.001$) (2-way ANOVA, $n = 14$ CFSS and 11 sham FS, **Fig. 1B**). In addition, as evaluated by the FST, the stressed rats robustly spent a longer time immobile (116.40 ± 21.35 seconds) than the control animals (27.82 ± 6.25 seconds) ($P < 0.001$, 2-tailed unpaired t test, $n = 11$ per group, **Fig. 1C**).

To determine whether chronic exposure of FS could also produce anxiety-like behavior in rats, we performed EPM test, which has been extensively used as a measurement of anxiety-like behavior in animal models.[12,52,68] The results revealed that there were no significant differences in the number of open arms entries (3.91 ± 0.58 CFSS vs 3.70 ± 0.26 sham FS) as well as in the percentage of the time spent in open arms (21.17 ± 2.81% CFSS vs 24.77 ± 2.65% sham FS) and the number of total arms entries (9.09 ± 1.10 CFSS vs 9.10 ± 0.62 sham FS) between the stressed rats and the control animals ($P > 0.05$, 2-way ANOVA, $n = 11$ CFSS and 10 sham FS, **Fig. 1D to F**). These data imply that CFSS cannot induce anxiety-like behavior in rats, as assessed by the EPM test.

Next, we examined effects of CFSS on nerve injury–induced mechanical allodynia in neuropathic rats. We found that CFSS resulted in an exacerbation of mechanical allodynia in rats subjected to SNI surgery (**Fig. 1G**). In nonstressed animals, pain allodynia was indeed observed following SNI as previously reported.[11] The ipsilateral PWT (in grams) was statistically decreased from day 1 (3.76 ± 0.42 SNI vs 12.46 ± 1.07 sham SNI) to day 14 (2.47 ± 0.41 SNI vs 14.51 ± 0.37 sham SNI) after SNI surgery as compared with sham surgery ($P < 0.001$, 2-way ANOVA, $n = 11$ per group). However, in stressed rats, this SNI-induced decrease of PWT was more reduced when the animal received chronic FS preconditioning to SNI surgery (0.69 ± 0.19 CFSS + SNI vs 3.31 ± 0.38 sham FS + SNI on day 1 post-SNI surgery, $P < 0.05$; 0.39 ± 0.16 CFSS + SNI vs 3.09 ± 0.46 sham FS + SNI on day 10 post-SNI surgery, $P < 0.05$) (2-way ANOVA, $n = 8$-12 per group). In contrast, pre-exposure of CFSS had no significant effect on the PWT in sham surgery rats ($P > 0.05$, CFSS + sham SNI vs sham SNI, 2-way ANOVA, $n = 11$ per group). Likewise, in the absence of nerve injury, CFSS

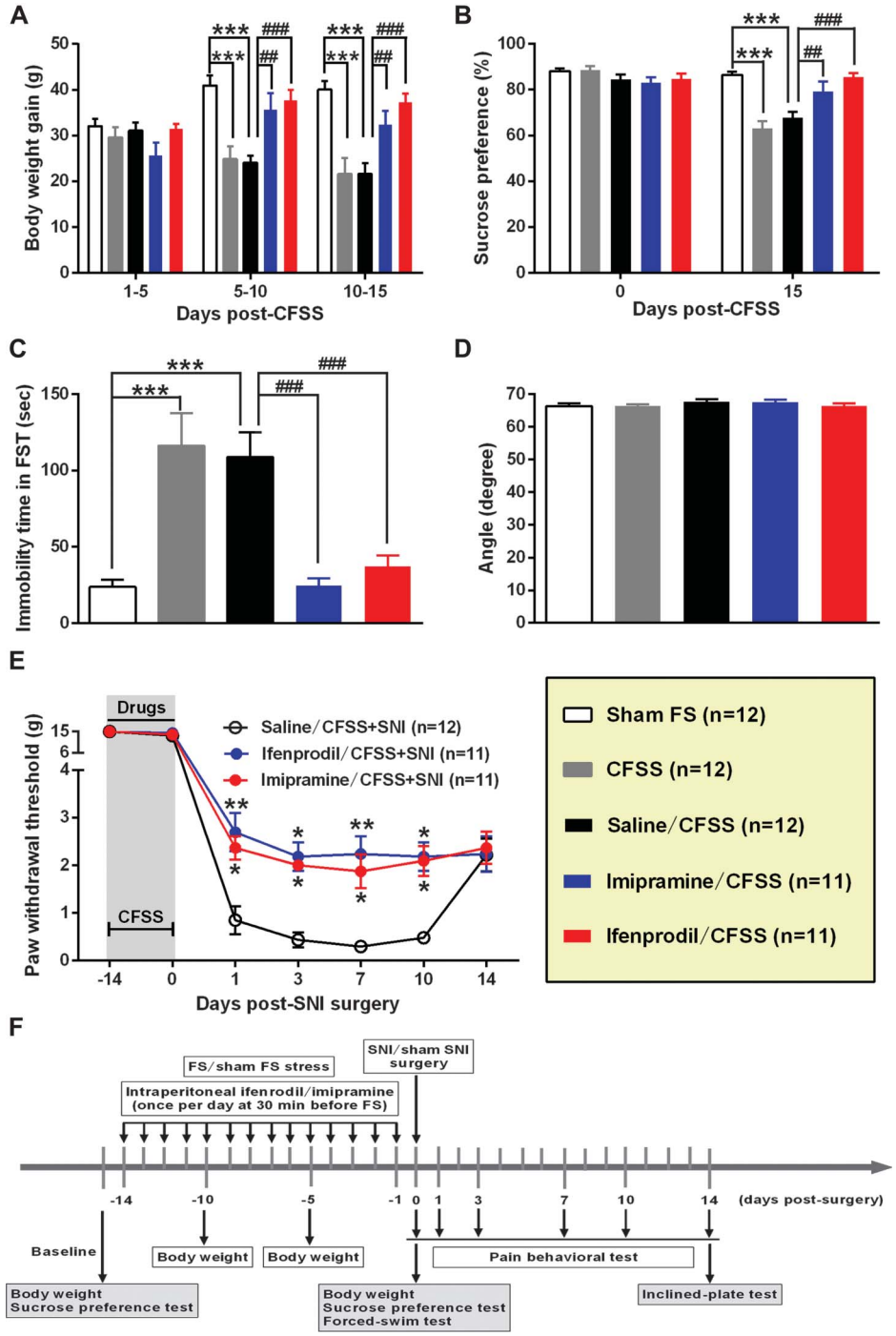

Figure 9. Effects of concomitant administration of the antidepressants, imipramine or ifenprodil, on chronic forced swimming (FS)-induced depressive-like behaviors in stressed rats, and on chronic FS-induced exacerbation of pain allodynia in stress-treated spared nerve injury (SNI) rats. (A–C) Assessment of depressive-like behaviors. Note that both imipramine and ifenprodil robustly exerted an antidepressant-like effect on stress-treated rats, as indicated by a rescue for the decreased body weight gains (A), the reduced preference for sucrose consumption (B), and the increased immobility time during the forced swim tests (C) following exposure of chronic forced swimming stress (CFSS). ***$P < 0.001$, ##$P < 0.01$, ###$P < 0.001$, 2-way or 1-way analysis of variance (ANOVA), $n = 11$ to 12 per group. (D) Assessment of locomotor function. No significant motor dysfunction is found in rats administered with imipramine or ifenprodil, as assessed by inclined-plate test. $P > 0.05$, 1-way ANOVA, $n = 11$ to 12 per group. (E) Assessment of mechanical allodynia. Note that the decreased paw withdrawal threshold is significantly reversed by chronic administration of either imipramine or ifenprodil in SNI rats subject to chronic FS preconditioning. *$P < 0.05$, **$P < 0.01$, 2-way ANOVA, $n = 11$ to 12 per group. (F) Schematic representation of the experimental procedure.

alone did not affect the PWT in intact rats ($P > 0.05$, CFSS vs sham FS, 2-way ANOVA, $n = 10$ per group). Moreover, as assessed by inclined-plate test, no significant motor dysfunction was found in rats subjected to CFSS, SNI surgery, or CFSS plus SNI surgery ($P > 0.05$, 1-way ANOVA, $n = 8$-12 per group, **Fig. 1H**).

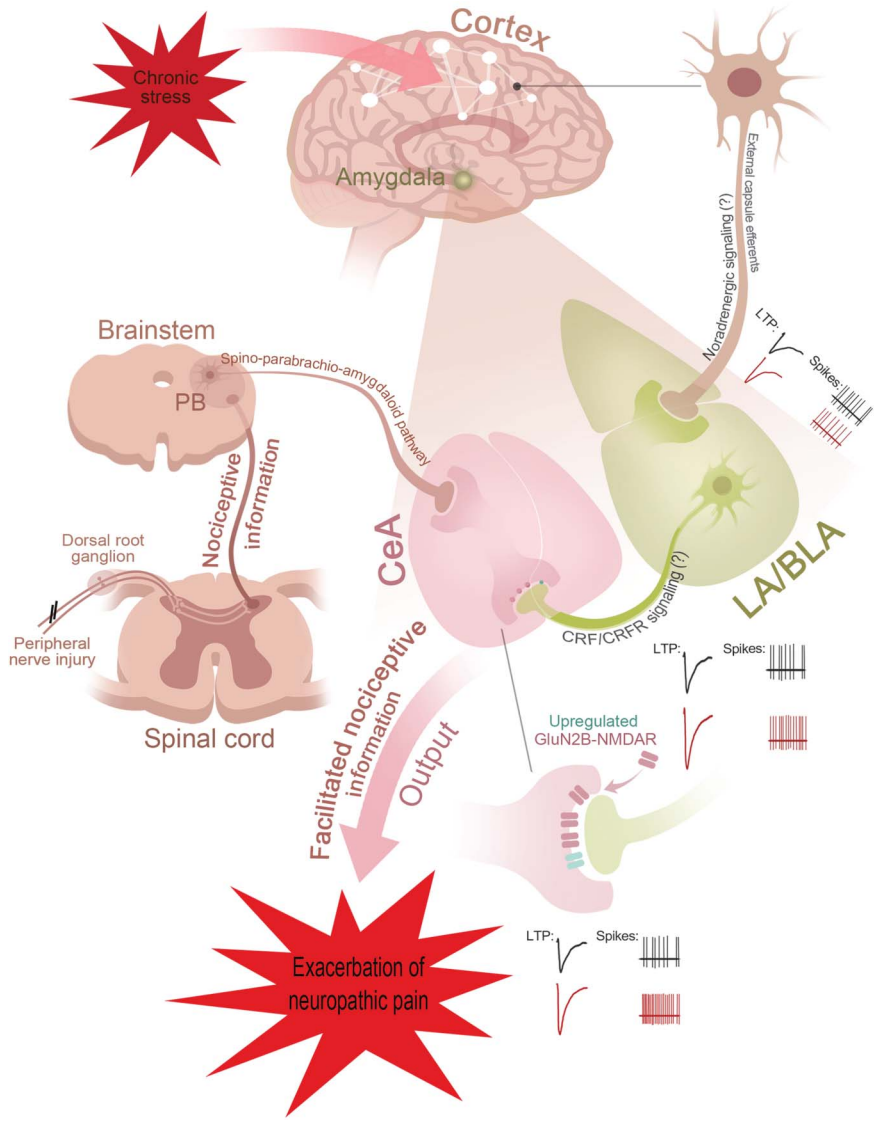

Figure 10. Schematic illustration of the possible mechanisms underlying chronic stress-induced exacerbation of neuropathic pain. In spared nerve injury (SNI) rats subjected to chronic stress preconditioning, 2 lines of input to the CeA including the parabrachial (PB)–central nucleus of the amygdala (CeA) synapse and the basolateral amygdala (BLA)-CeA synapse are activated. The nociceptive-specific information results from peripheral nerve injury (eg, SNI surgery) reaches to the CeA directly from the PB area through the spino-parabrachio-amygdaloid pain pathway, to enhance the neuronal activity in the CeA and potentiate synaptic plasticity at the PB-CeA synapse by up-regulating GluN2B-NMDA receptors. However, the information of chronic stress reaches to the lateral amygdala (LA)/BLA indirectly via thalamic and cortical afferents coursing into the region from the external capsule (EC), to augment the neuronal activity in the BLA and exaggerate synaptic plasticity at the BLA-CeA synapse by the activation of putative noradrenergic signaling and corticotropin-releasing factor (CRF) signaling, respectively (from our unpublished data). Here, the highly integrated stress-affect–related information is then transmitted to the CeA, which subsequently enhances the upregulation of GluN2B-NMDA receptors and increase the sensitization of CeA neurons, thereby facilitating pain-related synaptic plasticity of the PB-CeA pathway and exacerbating the nerve injury–induced neuropathic pain.

3.2. Chronic forced swim stress induces both sensitization of basolateral amygdala neurons and augmentation of long-term potentiation at the basolateral amygdala–central nucleus of the amygdala synapse

The lateral and basolateral subdivisions of the amygdala (BLA) form the primary input nuclei in the amygdala's emotion-related neural circuitry[45] and receive polymodal sensory information via cortical afferents coursing into the region from the EC.[89] This processed, polymodal information including stressful and affective information subsequently reaches the CeA, thereby regulating the nociceptive-specific information that is directly received from the pontine PB nucleus through the spino-parabrachio-amygdaloid pain pathway.[100] To test this hypothesis, we first examined the electrically evoked responses of BLA neurons by the stimulation of EC (EC). We found that chronic exposure of FS stress to intact rats caused a remarkable increase in the evoked discharges of BLA neurons responding to electrical stimulation of the EC (**Fig. 2A, B and G, H**). The averaged electrically evoked responses (spikes count) of BLA neurons during 1 hour were statistically increased in the CFSS-treated rats (9.02 ± 0.20) as compared with the sham FS rats (5.13 ± 0.05) ($P < 0.001$, 1-way ANOVA, $n = 17$ CFSS and 14 sham FS, **Fig. 2G and H**). Unexpectedly, SNI surgery had no significant influence on the evoked discharges of BLA neurons (average: 5.63 ± 0.13 SNI vs 4.40 ± 0.12 sham SNI, $P > 0.05$, 1-way ANOVA, $n = 13$ SNI and 11 sham SNI, **Fig. 2C, D and G, H**). However, pre-exposure of

chronic FS to SNI rats could produce a more significant increase in evoked discharges of BLA neurons in the CFSS + SNI rats (15.84 ± 0.25) as compared with the sham FS + SNI rats (5.48 ± 0.12, $P < 0.001$) as well as the CFSS rats (9.02 ± 0.20, $P < 0.001$) and the SNI rats (5.63 ± 0.13, $P < 0.001$), respectively (1-way ANOVA, $n = 11$ CFSS+SNI, 12 sham FS + SNI, 17 CFSS, and 13 SNI, **Fig. 2E, F and G, H**). These results indicate that chronic exposure of FS to rats not only enhances the electrically evoked responses of BLA neurons to the EC stimulation but also facilitates the effects of nerve injury on the evoked discharges of BLA neurons.

Furthermore, we investigated the spontaneous activity of BLA neurons that were identified as output neurons projecting to the CeA. We found that chronic exposure of FS to intact rats could result in a statistical increase in spontaneous activity (spikes per second) of BLA neurons responding to both innocuous and noxious mechanical stimuli (smooth forceps: 12.46 ± 1.86 CFSS vs 5.93 ± 0.92 sham FS, $P < 0.05$; toothed forceps: 17.64 ± 2.05 CFSS vs 8.47 ± 1.12 sham FS, $P < 0.001$, 2-way ANOVA, $n = 22$ CFSS and 17 sham FS, **Fig. 3A, B and G, H**). Similarly, SNI surgery alone had no significant influence on the spontaneous discharges of BLA neurons (smooth forceps: 6.90 ± 0.85 SNI vs 5.34 ± 0.61 sham SNI, $P > 0.05$; toothed forceps: 9.67 ± 1.19 SNI vs 6.97 ± 0.61 sham SNI, $P > 0.05$, 2-way ANOVA, $n =$ 19 SNI and 24 sham SNI, **Fig. 3C, D and G, H**). However, pre-exposure of chronic FS to SNI rats produced a more significant increase in spontaneous discharges of BLA neurons in the CFSS + SNI rats (smooth forceps: 21.61 ± 3.29; toothed forceps: 26.31 ± 3.69) as compared with the sham FS + SNI rats (smooth forceps: 7.81 ± 0.97; toothed forceps: 10.36 ± 1.33, $P < 0.001$) as well as the CFSS rats (smooth forceps: 12.46 ± 1.86; toothed forceps: 17.64 ± 2.05, $P < 0.001$) and the SNI rats (smooth forceps: 6.90 ± 0.85; toothed forceps: 9.67 ± 1.19, $P < 0.001$), respectively (2-way ANOVA, $n = 24$ CFSS + SNI, 17 sham FS + SNI, 22 CFSS, and 19 SNI, **Fig. 3E, F and G, H**). These data indicate that chronic exposure of FS to rats also enhances the spontaneous activity of BLA neurons and facilitates the effects of nerve injury on the spontaneous discharges of BLA neurons.

Moreover, we explored the LTP of fEPSPs at the BLA-CeA synapse. The results revealed that chronic exposure of FS to intact rats could induce a notable augmentation of LTP at the BLA-CeA synapse. When compared to the baseline responses (averaged at 0-20 minutes before HFS), the mean amplitude of fEPSPs after HFS was 145.5 ± 1.72% in sham FS rats and 171.0 ± 1.49% in CFSS rats ($P < 0.001$, vs sham FS group, 2-tailed unpaired *t* test, $n = 7$ sham FS and 6 CFSS, **Fig. 4A**). However, SNI surgery had no significant influence on the LTP of fEPSPs at the BLA-CeA synapse. For example, the mean amplitude of fEPSPs after HFS was 143.7 ± 1.20% of baseline in sham SNI rats and 146.8 ± 1.41% of baseline in SNI rats ($P > 0.05$, vs sham SNI group, 2-tailed unpaired *t* test, $n = 5$ per group, **Fig. 4B**). In contrast, pre-exposure of chronic FS to SNI rats produced a more significant increase in the LTP of fEPSPs at the BLA-CeA synapse. The mean amplitude of fEPSPs after HFS was significantly increased from 139.4 ± 2.11% of baseline in sham FS + SNI rats and 146.8 ± 1.41% of baseline in SNI rats to 173.3 ± 1.91% of baseline in CFSS + SNI rats ($P < 0.001$, vs sham FS + SNI group and SNI group, respectively, 1-way ANOVA, $n =$ 5-6 per group, **Fig. 4C and D**). Together with these data and aforementioned findings, we suggest that chronic exposure of FS to rats not only causes sensitization of BLA neurons but also induces augmentation of LTP at the BLA-CeA synapse, thereby exacerbating the effects of nerve injury on both BLA neurons activity and the transmission efficiency of the BLA-CeA synapse.

3 .3. Chronic forced swim stress exacerbates both the nerve injury–induced sensitization of central nucleus of the amygdala neurons and the long-term potentiation of field excitatory postsynaptic potentials at the parabrachial-central nucleus of the amygdala synapse

Pain-related synaptic plasticity and sensitization of CeA neurons has been shown in animal models of inflammatory pain[72,73] and neuropathic pain.[40,52] To test whether pain-related plasticity in CeA is the consequence of converging stress-affect–related input from BLA with nociceptive input from spinal cord and brainstem,[70,74] we examined effects of CFSS on nerve injury–induced sensitization of CeA neurons and LTP at the PB-CeA synapse. The results showed that both CFSS and SNI surgery could enhance the spontaneous activity of MR neurons in the CeA responding to innocuous (brush) and noxious (pinch) mechanical stimuli. For example, the stimulus–responses of MR neurons (spikes per second) were significantly increased in the CFSS-treated rats (innocuous: 4.55 ± 1.12; noxious: 7.72 ± 1.54) as compared with the sham FS-treated rats (innocuous: 1.14 ± 0.18; noxious: 2.53 ± 0.29) ($P < 0.001$, 2-way ANOVA, $n = 14$ CFSS and 15 sham FS, **Fig. 5A, B and H**). Similarly, the stimulus–responses of MR neurons (spikes per second) were statistically increased also in SNI rats (innocuous: 6.22 ± 0.67; noxious: 8.60 ± 0.80) as compared with sham SNI rats (innocuous: 1.39 ± 0.17; noxious: 2.27 ± 0.37) ($P < 0.001$, 2-way ANOVA, $n = 13$ SNI and 11 sham SNI, **Fig. 5C, D and H**). Moreover, SNI surgery could also cause a prominent increase in the baseline activity (spikes per second) of MR neurons (4.15 ± 0.65 SNI vs 0.66 ± 0.10 sham SNI, $P < 0.01$, 2-way ANOVA, **Fig. 5H**). As our expectation, pre-exposure of chronic FS to SNI rats produced a more significant increase in the stimulus–responses and the baseline activity of MR neurons. For instance, the stimulus–responses of MR neurons (spikes per second) were markedly increased in CFSS + SNI rats (innocuous: 10.28 ± 0.75; noxious: 13.32 ± 0.92) as compared with the sham FS +SNI rats (innocuous: 6.15 ± 0.41; noxious: 8.48 ± 0.47, $P < 0.001$) and the CFSS + sham SNI rats (innocuous: 4.35 ± 0.50; noxious: 6.99 ± 1.16, $P < 0.001$); likewise, the baseline activity of MR neurons (spikes per second) also was enhanced in the CFSS + SNI rats (7.73 ± 0.77) as compared with the sham FS + SNI rats (4.17 ± 0.39, $P < 0.01$) and the CFSS + sham SNI rats (2.17 ± 0.28, $P <$ 0.001) (2-way ANOVA, $n = 15$ CFSS + SNI and 11 sham FS + SNI and 10 CFSS + sham SNI, **Fig. 5E to H**). These results suggest that chronic exposure of FS to rats also enhances the stimulus–responses of MR neurons and potentiates the nerve injury–induced sensitization of MR neurons in the CeA.

With respect to the LTP at the PB-CeA synapse, we found that both chronic FS and SNI surgery could enhance the LTP of fEPSPs at the PB-CeA synapse. For example, when compared to the baseline responses (averaged at 0-20 minutes before HFS), the mean amplitude of fEPSPs after HFS was statistically increased from 139.1 ± 2.80% in sham FS rats to 156.3 ± 2.22% in CFSS rats ($P < 0.001$, vs sham FS group, 2-tailed unpaired *t* test, $n = 6$ sham FS and 7 CFSS, **Fig. 6A**) and from 137.9 ± 2.48% in sham SNI rats to 166.8 ± 3.38% in SNI rats ($P < 0.001$, vs sham FS group, 2-tailed unpaired *t* test, $n = 5$ sham SNI and 6 SNI, **Fig. 6B**), respectively. Moreover, pre-exposure of chronic FS to SNI rats also produced a more significant increase in the LTP of fEPSPs at the PB-CeA synapse. When compared to the baseline responses, the mean amplitude of fEPSPs after HFS was prominently increased from 166.0 ± 3.06% in sham FS + SNI rats and 154.8 ± 2.38% in CFSS + sham SNI rats to 191.6 ± 2.30% in CFSS + SNI rats ($P < 0.001$, vs sham FS + SNI group and CFSS + sham SNI group, respectively,

one-way ANOVA, $n = 5$-7 per group, **Fig. 6C and D**). These data suggest that chronic exposure of FS to rats not only exacerbates the nerve injury–induced sensitization of MR neurons in the CeA but also enhances the LTP of fEPSPs at the PB-CeA synapse.

3.4. The GluN2B-containing NMDA receptor is required for chronic forced swim stress–induced augmentation of long-term potentiation at the parabrachial-central nucleus of the amygdala synapse and the exacerbation of pain allodynia in spared nerve injury rats

It has been established that the GluN2B-NMDA receptors play an important role in amygdaloid synaptic plasticity[64,67,99] and in several models of chronic pain.[16,39,84,85,112,117] We hence speculate that the GluN2B-NMDA receptor is probably involved in the augmentation of LTP at the PB-CeA synapse and the exacerbation of pain allodynia in SNI rats induced by CFSS. In fact, we found that Ro 25-6981, a specific antagonist of GluN2B-NMDA receptors, not only inhibited the CFSS-induced and the SNI-induced LTP at the PB-CeA synapse but also blocked the CFSS-induced augmentation of LTP at the PB-CeA synapse in SNI rats. For instance, when compared to the baseline responses, the mean amplitude of fEPSPs following HFS was statistically decreased from 152.1 ± 1.83% in saline/CFSS rats to 113.9 ± 1.04% in Ro/CFSS rats ($P < 0.001$) and from 167.0 ± 3.03% in saline/SNI rats to 114.8 ± 1.21% in Ro/SNI rats ($P < 0.001$), respectively (2-tailed unpaired t test, $n = 5$-7 per group, **Fig. 7A and B**). Similarly, the mean amplitude of fEPSPs after HFS was more decreased from 193.3 ± 2.58% in saline/CFSS + SNI rats to 115.8 ± 1.73% in Ro/CFSS + SNI rats ($P < 0.001$, 2-tailed unpaired t test, $n = 6$ per group, **Fig. 7C**). In addition, we observed that intra-CeA administration of ifenprodil, another antagonist of GluN2B-NMDA receptors that has a longer effect than Ro 25-6981,[85] not only alleviated the nerve injury–induced mechanical allodynia in SNI rats but also inhibited the CFSS-induced exacerbation of neuropathic pain in stress-treated SNI rats. As shown in **Figure 7D**, in nonstressed animals, the SNI-induced decrease in ipsilateral PWT (in gram) was statistically reversed by the intra-CeA administration of ifenprodil from day 4 (4.10 ± 0.43 ifenprodil/SNI vs 2.24 ± 0.36 vehicle/SNI, $P < 0.001$) to day 6 (4.40 ± 0.58 ifenprodil/SNI vs 2.21 ± 0.34 vehicle/SNI, $P < 0.001$) post-SNI surgery (2-way ANOVA, $n = 10$ ifenprodil/SNI and 9 vehicle/SNI). In stressed animals, intra-CeA administration of ifenprodil also could inhibit the CFSS-induced decrease in ipsilateral PWT from day 4 (2.20 ± 0.33 ifenprodil/CFSS + SNI vs 0.54 ± 0.19 vehicle/CFSS + SNI, $P < 0.01$) to day 6 (2.66 ± 0.47 ifenprodil/CFSS + SNI vs 1.04 ± 0.18 vehicle/CFSS + SNI, $P < 0.01$) post-SNI surgery in ifenprodil/CFSS + SNI rats as compared with vehicle/CFSS + SNI rats (2-way ANOVA, $n = 10$ per group). Taken together, these data indicate that the GluN2B-NMDA receptor is required for chronic FS-induced augmentation of LTP at the PB-CeA synapse and the exacerbation of pain allodynia in stress-treated SNI rats.

3.5. Chronic forced swim stress enhances the nerve injury–induced functional up-regulation of GluN2B-containing NMDA receptors in the central nucleus of the amygdala of spared nerve injury rats

To further provide direct evidence for the idea that the increased GluN2B-NMDA receptors contribute to the chronic FS-induced augmentation of LTP at the PB-CeA synapse and the exacerbation of pain allodynia in stress-treated SNI rats, we examined the receptors' expression in synaptosomal fraction extracted from the right CeA tissue, which is usually performed to assess functional expression of receptors at a synapse.[26,79] As shown in **Figure 8**, in nonstressed animals, SNI surgery produced a significant increase in expression of GluN1 (1.16 ± 0.08 SNI vs 0.77 ± 0.07 sham SNI, $P < 0.01$, **Fig. 8A**), GluN2B (1.20 ± 0.05 SNI vs 0.89 ± 0.08 sham SNI, $P < 0.01$, **Fig. 8B**), and the ratio of GluN2B/GluN2A (1.13 ± 0.06 SNI vs 0.84 ± 0.13 sham SNI, $P < 0.05$, **Fig. 8D**) in synaptosomal fraction of the CeA on day 7 postsurgery (1-way ANOVA, $n = 4$ per group). In stressed animals, although exposure of intact rats to chronic FS could not affect the synaptic expression of GluN1, GluN2A, and GluN2B in the CeA ($P > 0.05$, **Fig. 8A–C**), pre-exposure of chronic FS to SNI rats resulted in a significant increase in the SNI-induced up-regulation of GluN1 (1.47 ± 0.07 CFSS + SNI vs 1.21 ± 0.09 sham FS + SNI vs 0.91 ± 0.12 CFSS + sham SNI, $P < 0.05$-0.001), GluN2B (1.37 ± 0.03 CFSS + SNI vs 1.22 ± 0.06 sham FS + SNI vs 0.75 ± 0.09 CFSS + sham SNI, $P < 0.05$-0.001), and the ratio of GluN2B/GluN2A (1.46 ± 0.07 CFSS + SNI vs 1.23 ± 0.06 sham FS + SNI vs 0.92 ± 0.12 CFSS + sham SNI, $P < 0.05$-0.01) in synaptosomal fraction of the CeA (1-way ANOVA, $n = 4$ per group). Similar potentiation of CFSS to SNI-induced up-regulation of GluN1 and GluN2B total protein expression as well as the ratio of GluN2B/GluN2A also were observed on the right CeA rather than the left CeA (supplementary data: Fig. S2, available online at http://links.lww.com/PAIN/A378). These results suggest that CFSS enhances the nerve injury–induced functional up-regulation of GluN2B-NMDA receptors in the CeA of SNI rats.

3.6. Concomitant administration of antidepressants inhibits the chronic forced swim stress–elicited depressive-like behaviors and the exacerbation of nerve injury–induced mechanical allodynia in rats

Finally, to determine whether stress-related depression underlies the chronic FS-induced exacerbation of pain hypersensitivity in neuropathic rats, we investigated effects of antidepressants including imipramine and ifenprodil on chronic FS-induced depressive-like behaviors in stressed animals and on the exacerbation of SNI-induced mechanical allodynia in stress-treated SNI rats. In agreement with previous findings, chronic administration of either imipramine or ifenprodil robustly displayed an antidepressant-like effect on chronic FS-stressed rats. For instance, the decreased body weight gains during days 5 to 10 (35.64 ± 3.63 g imipramine/CFSS and 37.64 ± 2.31 g ifenprodil/CFSS vs 24.08 ± 1.55 g saline/CFSS, $P < 0.01$-0.001) and days 10 to 15 (32.46 ± 2.91 g imipramine/CFSS and 37.27 ± 1.87 g ifenprodil/CFSS vs 21.58 ± 2.45 g saline/CFSS, $P < 0.01$-0.001) induced by CFSS was effectively blocked by chronic administration of either imipramine or ifenprodil (2-way ANOVA, $n = 11$-12 per group, **Fig. 9A**). Moreover, chronic administration of both the antidepressants also could inhibit the chronic FS-induced reduction of sucrose solution intake (79.21 ± 4.39% imipramine/CFSS and 85.46 ± 1.76% ifenprodil/CFSS vs 67.80 ± 2.46% saline/CFSS, $P < 0.01$-0.001) (2-way ANOVA, **Fig. 9B**) and the prolonged immobility in chronic FS-stressed rats (24.55 ± 5.06 seconds imipramine/CFSS and 37.17 ± 7.21 seconds ifenprodil/CFSS vs 108.80 ± 16.31 seconds saline/CFSS, $P < 0.001$) (1-way ANOVA, **Fig. 9C**). More importantly, the chronic FS-induced pain exacerbation in stress-treated SNI rats also was significantly reversed by both imipramine and ifenprodil. The decreased PWT in chronic FS-stressed SNI rats was prominently restored from day 1 (2.36 ± 0.24 g imipramine/

CFSS + SNI and 2.69 ± 0.40 g ifenprodil/CFSS + SNI vs 0.85 ± 0.30 g saline/CFSS + SNI, $P < 0.05$-0.01) to day 10 (2.09 ± 0.32 g imipramine/CFSS + SNI and 2.18 ± 0.30 g ifenprodil/CFSS + SNI vs 0.48 ± 0.11 g saline/CFSS + SNI, $P < 0.05$) by chronic administration of both the antidepressants (2-way ANOVA, **Fig. 9E**). In addition, as assessed by inclined-plate test, no significant motor dysfunction was found in rats subjected to CFSS surgery or received chronic administration of both the antidepressants ($P > 0.05$, 1-way ANOVA, **Fig. 9D**). Taken together, these data suggest that the stress-related depression plays a crucial role in the chronic FS-induced exacerbation of pain hypersensitivity in neuropathic rats.

To determine whether the analgesic effects of the antidepressants are specific to the CFSS-induced allodynia and whether the commonly used analgesic has an efficacy to the CFSS-induced exacerbation of chronic pain, we performed additional 2 experiments to examine: (1) effects of the antidepressants imipramine or ifenprodil on pain behaviors in SNI rats and (2) effects of indomethacin (Indo), a NSAID, on CFSS-induced exacerbation of pain allodynia in stress-treated SNI (CFSS + SNI) rats (supplementary data: Fig. S3, available online at http://links.lww.com/PAIN/A378). As shown in Figure S3A (http://links.lww.com/PAIN/A378), when administered i.p. to nonstressed SNI rats, both the antidepressants imipramine (10 mg·kg^{-1}) and ifenprodil (3 mg·kg^{-1}) only have a transient analgesic effect as compared with the vehicle group. Although imipramine displayed a weak analgesic effect as indicated by a decreased PWT on day 1 (8.22 ± 1.87 g imipramine vs 4.44 ± 1.56 g vehicle) and day 3 (8.22 ± 1.87 g imipramine vs 4.44 ± 1.56 g vehicle) postsurgery ($P < 0.05$, 2-way ANOVA, $n = 9$ per group), ifenprodil only showed a transient analgesic effect on day 1 postsurgery (9.29 ± 2.14 g ifenprodil vs 4.44 ± 1.56 g vehicle, $P < 0.01$, 2-way ANOVA, $n =$ 7-9 per group). In stress-treated SNI (CFSS + SNI) rats, both low dose (5 mg·kg^{-1}) and high dose (10 mg·kg^{-1}) of indomethacin (Indo) have not any analgesic effect when i.p. administered to rats in a pretreatment manner (ie, during chronic FS preconditioning) ($P > 0.05$, compared to vehicle, 2-way ANOVA, $n = 7$-12 per group, Fig. S3B, http://links.lww.com/PAIN/A378). However, when administered in a posttreatment manner (ie, after chronic FS preconditioning), despite low dose (5 mg·kg^{-1}) of Indo still has not obvious analgesic effect, high dose (10 mg·kg^{-1}) of Indo indeed displayed a significant analgesic effect to stress-treated SNI rats (PWT, 4.39 ± 0.55 g Indo vs 1.38 ± 0.18 g vehicle on day 1 postsurgery; 2.34 ± 0.60 g Indo vs 0.38 ± 0.12 g vehicle on day 3 postsurgery) ($P < 0.001$, 2-way ANOVA, $n = 8$-13 per group, Fig. S3C, http://links.lww.com/PAIN/A378). As assessed by inclined-plate test, no significant motor dysfunction was found in rats that received each of the above drugs ($P > 0.05$, 1-way ANOVA, Fig. S3D, http://links.lww.com/PAIN/A378). These results suggest that the NSAIDs only have an analgesic effect to nerve injury–elicited pain hypersensitivity but cannot attenuate the chronic stress-induced exacerbation of neuropathic pain.

In addition, we conducted another additional experiment to determine whether intra-BLA administration of the antidepressants also has effects on chronic FS-induced depressive-like behaviors in stressed rats and on chronic FS-induced exacerbation of pain allodynia in stress-treated SNI rats (supplementary data: Fig. S4, available online at http://links.lww.com/PAIN/A378). We found that both the antidepressants including imipramine and ifenprodil, when intra-BLA administered to rats during the CFSS training, indeed produced a significant antidepressant-like effect on chronic FS-stressed rats, as indicated by an abrogation of chronic FS-induced reduction of sucrose preference (77.63 ± 4.45% imipramine/CFSS and 81.15 ± 3.97% ifenprodil/CFSS vs 50.67 ± 11.22% vehicle/CFSS, $P < 0.01$) (2-way ANOVA, Fig. S4B, http://links.lww.com/PAIN/A378), and the prolonged immobility in stress-treated rats (46.29 ± 7.15 seconds imipramine/CFSS and 50.73 ± 4.49 seconds ifenprodil/CFSS vs 86.02 ± 10.46 seconds vehicle/CFSS, $P < 0.01$) (1-way ANOVA, $n = 9$-11 per group, Fig. S4C, http://links.lww.com/PAIN/A378). Moreover, we found that both imipramine and ifenprodil, when intra-BLA administered to stress-treated SNI rats during the chronic FS preconditioning, could also abrogate the chronic FS-induced exacerbation of neuropathic pain, as indicated by a reversal of decreased PWT from day 1 (2.62 ± 0.71 g ifenprodil/CFSS + SNI vs 1.22 ± 0.38 g vehicle/CFSS + SNI, $P < 0.05$) to day 7 (1.42 ± 0.31 g imipramine/CFSS + SNI and 1.64 ± 0.43 g ifenprodil/CFSS + SNI vs 0.11 ± 0.02 g vehicle/CFSS + SNI, $P <$ 0.05-0.01) post-SNI surgery (2-way ANOVA, $n = 9$-11 per group, Fig. S4E, http://links.lww.com/PAIN/A378). Similarly, as assessed by inclined-plate test, no significant motor dysfunction was found in rats subjected to CFSS surgery or received chronic administration of both the antidepressants ($P > 0.05$, 1-way ANOVA, Fig. S4D, http://links.lww.com/PAIN/A378). These data suggested that inhibition of the depression development by intra-BLA administration of the antidepressants could also ameliorate the chronic FS-induced exacerbation of pain allodynia in stress-treated SNI rats. Thus, the stress-related depression is required for chronic FS-induced exacerbation of pain hypersensitivity in neuropathic rats.

4. Discussion

In this study, we found that exposure of chronic FS, which has been applied as a mixed psychological and physical stressor,[9,22,46] resulted in depressive-like behavior rather than anxiety-like behavior in stressed rats. Pre-exposure of SNI rats to CFSS induced an exacerbation of pain allodynia in neuropathic animals. These results are supported by previous findings that repeated or chronic exposure to stress typically causes stress-induced hyperalgesia in humans[20,34,47] and rodents.[4,17,41,42] Pain and negative emotions have the capacity to influence each other reciprocally.[100] The high comorbidity between chronic pain and mood disorders including anxiety and depression,[1,57,113] combined with the well-established link between chronic stress and mood disorders,[28,88] suggest an association with CFSS, depression, and chronic stress-induced exacerbation of neuropathic pain.[8] Although amount of data has shown that chronic stress can enhance hyperalgesia in humans and rodents associated with chronic pain, and even can increase pain sensitivity in healthy human,[20] our results showed that the basal nociception in sham rats was not significantly altered after CFSS. The reason for this discrepancy is probably due to the differences in experimental subjects (humans vs animals), gender (female subjects vs male rats), stressor (psychosocial stress vs forced swim), or times of stress (acute vs chronic). Of interest to note is that Crettaz et al.[20] found that this psychosocial stress only induces enhancement of pain sensitivity in response to thermal but not mechanical stimuli in healthy subjects. These results are in line with our findings that CFSS did not affect the basic nociceptive response to mechanical stimuli. In fact, accumulative evidence has documented that repeated FS stress indeed has no significant effect on mechanical sensitivity or even thermal sensitivity in intact rats,[41,42,46] in spite of the fact that it can enhance hyperalgesia in rats associated with inflammatory or neuropathic pain.

Among several nuclei of the amygdala, the CeA especially the CeLC receives nociceptive-specific information directly from the PB area through the spino-parabrachio-amygdaloid pain pathway,[5,30]

whereas the LA/BLA receive polymodal sensory information via cortical afferents coursing into the region from the EC[89] and form the primary input nuclei in the amygdala's emotion-related neural circuitry.[45] Here, the highly integrated stress-affective–related information is then transmitted to the CeA to mediate the stress/emotional modulation of persistent pain.[100] Consistent with literature findings that chronic stress induces hyperactivity and increased responsiveness to excitatory inputs of BLA neurons,[19,63,66,90,94,116] our results showed that CFSS indeed enhances the BLA neurons' activity and exaggerates LTP at the BLA-CeA synapse. These results are supported by previous findings that lesion of BLA inhibits the development of pain chronicity in neuropathic rats,[59] suggesting a role for BLA in the stress/emotional modulation of neuropathic pain. Of note, while SNI itself did not affect the neuronal activity in the BLA and LTP at the BLA-CeA synapse, it indeed affected the neuronal responses in the CeA and synaptic plasticity at the PB-CeA synapse. Both increased neuronal excitability in the CeA and enhanced synaptic transmission at the PB-CeA synapse are seen also in animal models of arthritic,[72,73] visceral,[38] and neuropathic pain.[40,52] Similarly, although enhanced synaptic transmission at the BLA-CeA synapse was observed in an arthritis model,[73] no significant alteration was found either in BLA neurons activity or in synaptic plasticity at the BLA-CeA synapse in models of visceral and neuropathic pain.[38,40] These data suggest that amygdala plasticity is specific for different types of inputs and pain states.[38]

However, a previous study has shown that while lesion of BLA was able to abolish the formalin-induced conditioned place aversion in rats, it failed to reduce the animals' nociceptive behaviors.[106] These results raise a possible explanation for our findings that despite the role for BLA in stress/emotional modulation of neuropathic pain, SNI itself did not affect the neuronal responses in the BLA. Together with aforementioned data showing that CFSS could enhance BLA neurons activity and LTP at the BLA-CeA synapse, we suggest that nociceptive-specific information results from peripheral nerve injury (eg, SNI surgery) reaches to the CeA directly from the PB area through the spino-parabrachio-amygdaloid pain pathway to enhance the neuronal activity in the CeA and potentiate synaptic plasticity at the PB-CeA synapse by up-regulating GluN2B-NMDA receptors. However, the information of CFSS reaches to the LA/BLA indirectly from thalamic and cortical areas, to augment the neuronal activity in the BLA and exaggerate synaptic plasticity at the BLA-CeA synapse, which subsequently enhances the upregulation of GluN2B-NMDA receptors and increase the sensitization of CeA neurons, thereby facilitating pain-related synaptic plasticity of the PB-CeA pathway and exacerbating the nerve injury–induced neuropathic pain (as shown in **Fig. 10**). Indeed, we found that pre-exposure of chronic FS to SNI rats exacerbates the effects of nerve injury on both BLA neurons' activity and synaptic plasticity of the BLA-CeA synapse.

In fact, we present multiple lines of evidence supporting the role of GluN2B-NMDA receptor in chronic FS-induced augmentation of LTP at the PB-CeA synapse and pain exacerbation in SNI rats. For instance, blockade of GluN2B-NMDA receptors with Ro 25-6981 inhibits the CFSS-induced augmentation of LTP at the PB-CeA synapse in SNI rats; intra-CeA administration of ifenprodil alleviates the CFSS-induced exacerbation of SNI-mediated neuropathic pain; also, pre-exposure of SNI rats to CFSS enhances the nerve injury–induced upregulation of synaptic GluN2B-NMDA receptors in the CeA. Surprisingly, our present results revealed that although CFSS enhanced the evoked response in CeA neurons, it did not affect the expression level of synaptic GluN1, GluN2A, and GluN2B in the CeA. These findings raise a possibility that the increased responsiveness of CeA neurons induced by CFSS is presumably through an indirect way that is different from the SNI-mediated sensitization of CeA neurons. Actually, we found that CFSS also induced an enhancement of BLA neurons activity and LTP at the BLA-CeA synapse. As stated above, we speculate that the information of chronic stress reaches to the BLA via thalamic and cortical afferents coursing into the region from EC.[89] Here, the highly integrated stress-affect–related information is then transmitted to the CeA and elicited the increased responsiveness of CeA neurons. This kind enhancement of CeA neurons activity induced by CFSS, differing from the SNI-induced sensitization of CeA neurons, is probably independent on the elevation of NMDA receptors. Activation of corticotropin-releasing factor signaling in the CeA, which has been shown to be involved in stress-induced sensitization of CeA neurons,[29,44,48,50] for example, likely mediates the increased responsiveness of CeA neurons after exposure to CFSS. In addition, chronic FS-induced elevation of GluN2B-NMDA expression in SNI rats appeared in the right CeA, but not the left CeA, supporting lateralization of amygdala function in pain and emotional processing,[2,14,36,51,115] eg, a dominant role for the right amygdala in the modulation of pain and pain-related negative emotions.[47,50–52]

Imipramine is a well-established tricyclic antidepressant that has been used clinically for many years.[95,108,110] Similar as the classic antidepressants, ifenprodil used chronically at a low dose by intraperitoneal administration also produces a significant antidepressant-like effect and therefore to be used here mainly as an antidepressant but not merely as a GluN2B-NMDA receptor antagonist.[32,33] In fact, when administered i.p. as described in previous studies,[32,35] both imipramine and ifenprodil have an antidepressant-like effect and can ameliorate chronic FS-induced exacerbation of neuropathic pain in stress-treated SNI rats. Although these antidepressants also have a transient and weak analgesic effect to nonstressed SNI rats, the underlying mechanisms between the former and the latter are probably different. With respect to the NSAIDs indomethacin, although it displays an analgesic effect to CFSS-induced pain exacerbation when i.p. administered after SNI surgery, no significant analgesic effect is observed when administered during FS preconditioning. Moreover, we provide additional evidence to show when specifically administered to the BLA, a relay station for transmitting stress-affect–related information to the CeA, both imipramine and ifenprodil also produce an antidepressant-like effect and can attenuate the CFSS-induced exacerbation of neuropathic pain. These data suggest that inhibition of stress-related depression by the antidepressants can effectively alleviate chronic FS-induced pain exacerbation in SNI rats subject to stress preconditioning. Therefore, the stress-related depression likely is an underlying contributor to chronic FS-induced exacerbation of neuropathic pain.

In conclusion, our findings provide solid evidence to show that chronic stress potentiates synaptic efficiency of the BLA-CeA pathway, leading to the activation of GluN2B-NMDA receptors and sensitization of CeA neurons, which subsequently facilitate pain-related synaptic plasticity of the PB-CeA pathway, thereby exacerbating nerve injury–induced neuropathic pain. We here present a novel understanding that chronic stress exacerbates neuropathic pain via the integration of stress-affect–related information with nociceptive information in the CeA.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplemental Digital Content

Supplemental Digital Content associated with this article can be found online at http://links.lww.com/PAIN/A378.

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